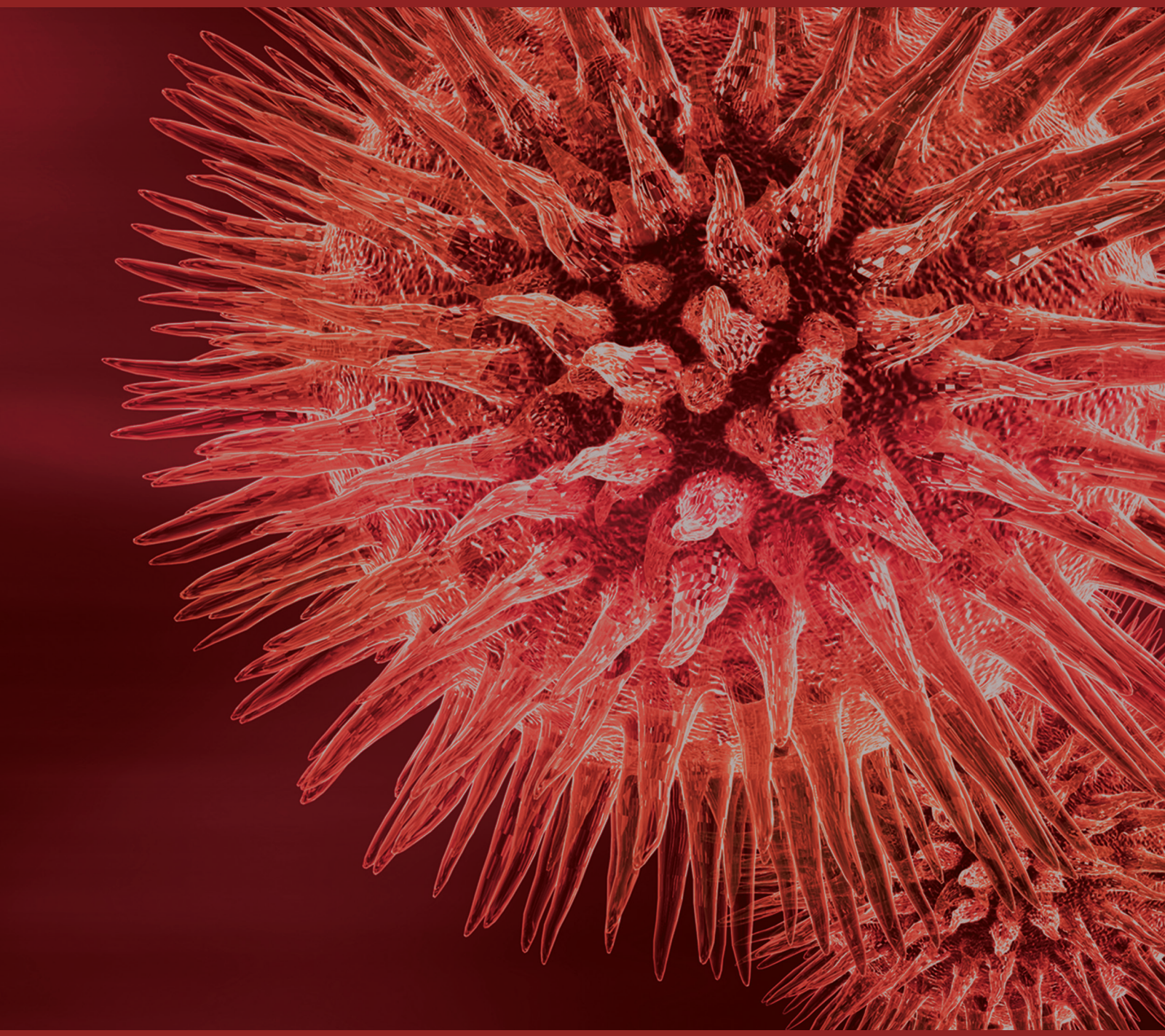
BioMed Research International

Cell Death in Human Health and Disease

Guest Editors: Jianzhen Xu, Dong Wang, and Wencai Ma



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Editorial

Cell Death in Human Health and Disease

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1. Introduction

Mammalian cells adopt several cellular mechanisms to die, including apoptosis, autophagy, and programmed necrosis. Cell death has an important homeostatic role, mediating the removal of damaged cells, organelles, and proteins. Accumulated studies have increasingly shown that cell death plays critical roles in a variety of human diseases including cancers, infection, and neurodegenerative diseases. Novel cell death based drug targets and predictive biomarkers have been evaluated in several clinical trials. This special issue summarizes some of the most recent advances in our understanding of the relationships between cell death pathways and human diseases.

2. Understanding the Molecular Mechanisms of Cell Death in Human Diseases

TNF-α induced neuroinflammation and neurotoxicity have been implicated in neurodegenerative diseases. However, the molecular mechanisms that lead to cytokine initiated neurotoxicity are not fully understood. Based on RIP3-deficient mice, S. Liu et al. reported that RIP3-mediated necroptosis is activated in the mouse hippocampus after intracerebroventricular injection of TNF-α. They demonstrated that the signaling pathway CYLD-RIP1-RIP3-MLKL plays an important role in TNF-α induced necrosis, while neither ROS accumulation nor calcium influx is critical for the execution of cell death. Their work provided the first *in vivo* evidence for a role of RIP3 in TNF-α induced neurotoxicity in hippocampal neurons.

Dysregulation of cell death pathway is one of the hallmarks of cancers. In osteosarcoma U2OS cells, P. Chen et al. found that estrogen-related receptor alpha (ERR) blocked methotrexate-induced cell death and attenuated the activation of p53-mediated apoptosis signaling pathway. Their results provided a novel molecular understanding of methotrexate resistance and identified potential treatment strategies for osteosarcoma.

Resveratrol has been used as a supplemental treatment for several neurological and nonneurological diseases. But it is not clear whether resveratrol has neuroprotective effect on amyotrophic lateral sclerosis (ALS), a fatal neurodegenerative disease. L. Song et al. found that resveratrol treatment attenuated motor neuron loss, reduced muscle atrophy, and improved mitochondrial function of muscle fibers in the ALS mice. Their findings suggested that resveratrol has antioxidant and antiapoptotic effects against ALS.

3. Summarizing the Recent Advances of Cell Death in Human Disease

In order to provide our readers with more comprehensive and deeper understanding of cell death in human diseases, we deliberately selected several nice review papers on this topic. In recent years, the fundamental regulatory roles of miRNAs have been linked to diverse physiological processes and human diseases. Focused on lung cancer, N. Othman and N. Hasima conducted a comprehensive survey about miRNAs in the apoptotic process and in sensitivity/resistance to common cancer treatments.

Cell death plays a multitiered immunological role in infection, inflammation, and immunity. In their review paper, T. Nunes et al. described the recent findings on the associations of cell death processes with the development of inflammatory bowel diseases in humans. They pointed out the complex crosstalk between autophagy, apoptosis, and necroptosis, which suggests that deeper understanding among the different forms of cell death is warranted.

Ubiquitination is a posttranslational modification in which ubiquitin is attached to proteins to modulate the degradation of substrate proteins via the proteasome and lysosome. Deubiquitinases (DUBs) regulate a variety of cellular processes by reversing ubiquitination. S. Bhattacharya and M. Ghosh summarized recent findings on the involvement of DUBs in cell death associated pathways related to cancers. This review provided a novel viewpoint of the regulation of cell death signaling.

Finally, our group contributed a review paper on cell death related biomarkers. We surveyed the current and emerging biomarkers involved in apoptosis, autophagy, and programmed necrosis and discussed their relationship with human diseases. This survey can help researchers to focus on the translation of laboratory discovery into clinical practice.

4. Prediction and Analysis of Cell Death Related Proteins and Gene Networks

Computational approaches play an increasing role in elucidating the components and molecular mechanisms of human cell death networks. J-proteins are molecular chaperones and can be classified into 4 types, that is, Type I, Type II, Type III, and Type IV. Different types of J-proteins play distinct roles in influencing cancer properties and cell death. Thus, reliably annotating the types of J-proteins is essential for better understanding of their molecular functions. P. Feng et al. proposed a support vector machine model to predict the four functional types of J-proteins based on reduced amino acid alphabet compositions. For the convenience of the experimental scientists, an online prediction server was also developed.

The formation and death of macrophages/foam cells are one of the major factors that cause coronary heart disease (CHD). Based on the Edinburgh human metabolic network (EHMN), X. Jia et al. built an enzyme-reaction network and proposed a computational approach to select modules related to programmed cell death. By integrating the subcellular location information with enzyme-reaction network, they identified the EHMN-mitochondria (EHMN-M) module, which was confirmed to be related to programmed cell death, CHD pathogenesis, and lipid metabolism via literature surveying. This method comprehensively analyzed CHD from the point of programmed cell death in subnetworks.

Acknowledgments

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Research Article

Identification of Modules Related to Programmed Cell Death in CHD Based on EHEN

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The formation and death of macrophages and foam cells are one of the major factors that cause coronary heart disease (CHD). In our study, based on the Edinburgh Human Metabolic Network (EHMN) metabolic network, we built an enzyme network which was constructed by enzymes (nodes) and reactions (edges) called the Edinburgh Human Enzyme Network (EHEN). By integrating the subcellular location information for the reactions and refining the protein-reaction relationships based on the location information, we proposed a computational approach to select modules related to programmed cell death. The identified module was in the EHEN-mitochondria (EHEN-M) and was confirmed to be related to programmed cell death, CHD pathogenesis, and lipid metabolism in the literature. We expected this method could analyze CHD better and more comprehensively from the point of programmed cell death in subnetworks.

1. Introduction

Coronary heart disease (CHD) is the narrowing or blockage of the coronary arteries, usually caused by atherosclerosis. Atherosclerosis is the buildup of plaques on the inner walls of the arteries, which can restrict blood flow to the heart muscle by physically clogging the artery or by causing abnormal artery tone and function [1]. In general, apoptosis occurring in atherosclerotic lesions has been suggested to be involved in the evolution and the structural stability of the plaques [2]. Because late apoptotic cells can launch proatherogenic inflammatory responses, adequate engulfment of apoptotic cells (efferocytosis) by macrophages is important to withstand the atherosclerosis progression [3]. Macrophages represent more than 40% of dead cells in the atherosclerotic lesions [4]. Enhancing intracellular lipid content can enlarge foam cells formation [5]. Formation of lipid-laden foam cells from macrophages and, to a less extent, from smooth muscle cells represents a landmark for atherosclerosis [6]. Some studies have documented that deregulation of apoptosis, a form of genetically programmed cell death, occurs in atherosclerotic lesions [7, 8].

Programmed cell death (PCD) is proposed to be death of a cell in pathological format, mediated by an intracellular program [9]. PCD referred to apoptosis, autophagy, and programmed necrosis. These three forms of PCD may jointly decide the fate of cells; apoptosis and programmed necrosis invariably contribute to cell death, whereas autophagy can play either prosurvival or prodeath roles [10]. Imbalance between cell survival and death may contribute to dramatic alterations in cellularity of the arterial wall with atherosclerosis. Carotid Atherosclerosis Progression Trial Investigating Vascular Cholesterol Acyltransferase (ACAT) Inhibition Treatment Effects was developed to assist in the prevention of cardiovascular disease [11]. Cyclooxygenase-2 (COX-2) in control of cell proliferation, apoptosis, played a significant role in the development of atherosclerosis [12, 13]. Matrix metalloproteinase produced by macrophages played

an important role in modulating plaque stability and apoptosis of cell [14, 15]. Macrophages play a crucial role at all stages of CHD, including regulation of foam cell formation, the inflammatory response, and the stability of atherosclerotic plaques. The effect of enzyme in macrophages and foam cells is of great value in the study of CHD from the perspective of programmed cell death.

Traditional experimental research was through single gene or single compound to analyze CHD, and it could not provide the analysis at system level. Along with the rapid development of technology, the study about biological networks is increasingly providing valuable information on biological systems [16–18]. Metabolic network is an important typical biochemical network which consists of enzymes and chemical compounds [19, 20]. Due to localization of metabolic enzymes, many metabolic processes involve coordinated interactions between different organelles, and one metabolic step may be dependent upon the successful completion of the previous step [21, 22]. In other words, chemical compounds can be considered as edges mediating between enzymes. A better understanding of human metabolism and its relationship with diseases is an important task in human systems biology studies. In metabolic reactions, there is a cascade relationship between enzymes. The relationship network between enzymes was constructed to select risk enzymes related disease and reveal the pathogenesis. Considering from this aspect, enzyme marker will be searched and be used for disease detection in the near future.

Therefore, in our paper, we presented a high-quality human metabolic enzyme network, Edinburgh Human Enzyme Network (EHEN), manually reconstructed based on Edinburgh Human Metabolic Network (EHMN) [23], and divide it into different subnetworks, by integrating genome annotation and location information from different databases and metabolic reaction information from the literature. We presented a strategy to select reporter enzyme considering the change of foam cells and macrophages between normal state and disease state with network characteristic. Through the method of selecting modules based reporter enzymes and the functional enrichment analysis, the module related to programmed cell death was analyzed further. We expected this method could analyze CHD better and more comprehensively from the point of programmed cell death in subnetworks.

2. Materials and Methods

2.1. Gene Expression Data Source and Data Preprocessing. We used monocyte-derived macrophages from peripheral blood cultured in the absence or presence of oxidized LDL, baseline macrophages or foam cells. The macrophages were obtained from 15 subjects with subclinical atherosclerosis and a family history of CHD. Macrophages from 15 age and gender matched subjects with no atherosclerosis and no family history of CHD were used as control. We downloaded this expression data with the accession number of GSE9874 [24] from NCBI-GEO database (http://www.ncbi.nlm.nih.gov/geo/).

2.2. Construction of Enzyme Network and Enzyme Subnetwork Based on EHMN. The Edinburgh Human Metabolic Network (EHMN) was reconstructed by integrating genome annotation information from different databases and metabolic reaction information from the literature [23]. EHMN contains nearly 3000 metabolic reactions, which were reorganized into 70 human-specific pathways according to their functional relationships [25, 26]. Based on EHMN metabolic network, we built an enzyme network which was constructed by enzyme (nodes) and reactions (edges). According to reaction relationships between substrates and products (i.e., the product of a reaction is just right the substrate of the next reaction), corresponding enzymes of reactions were connected to a network, as shown in Figure 1(a). Because the enzyme network construction is based on EHMN, the Edinburgh Human Metabolic Network, we called it the Edinburgh Human Enzyme Network (EHEN).

The enzyme protein location information in this work is extracted mainly from gene ontology (GO) [27]. Files containing GO association in human and the hierarchically organized GO terms ("OBO" file) can be easily downloaded from the GO website. This file is maintained by the GOA group at EBI which aims to provide high-quality GO annotations to proteins in the UniProtKB, as demonstrated in Figure 1(b). The proteins with uncertain locations or other locations not included in the known specific locations were classified to an "uncertain" location, and they will be finally screened out from our study.

2.3. Selection of Modules Related to Programmed Cell Death Based on Reporter Enzymes. We proposed a computational approach to select modules related to programmed cell death. All the disease and matched control samples were normalized simultaneously using the robust multiarray average (RMA) method [28], implemented in R/Bioconductor project. We calculated and obtained risk enzymes from the above expression profile of foam cells and macrophages, separately. Next, the common enzymes of two cells were sought as reporter enzymes. Further, the network functional modules related to programmed cell death based on reporter enzymes were selected. Details of our selection progress are as follows.

(1) P values of genes, P_value_g, indicating the significance of differential expression, were converted to standard Z-scores Z_value_g with a mean of 0 and a variance of 1 by using the inverse normal cumulative distribution function (CDF) (θ^{-1}):

$$Z\_\text{value}_g = \text{cdf}^{-1}\left(1 - P\_\text{value}_g\right). \quad (1)$$

(2) Z-scores of enzymes calculated as median of Z-scores of the k genes were as follows:

$$Z\_\text{value}_{\text{Enzyme}} = \frac{1}{k}\sum_{i=1}^{n}\left\{Z\_\text{value}_g\right\}. \quad (2)$$

(3) $Z\_\text{value}_{\text{Enzyme}}$ scores were then corrected for the background distribution by subtracting the mean (μ_k)

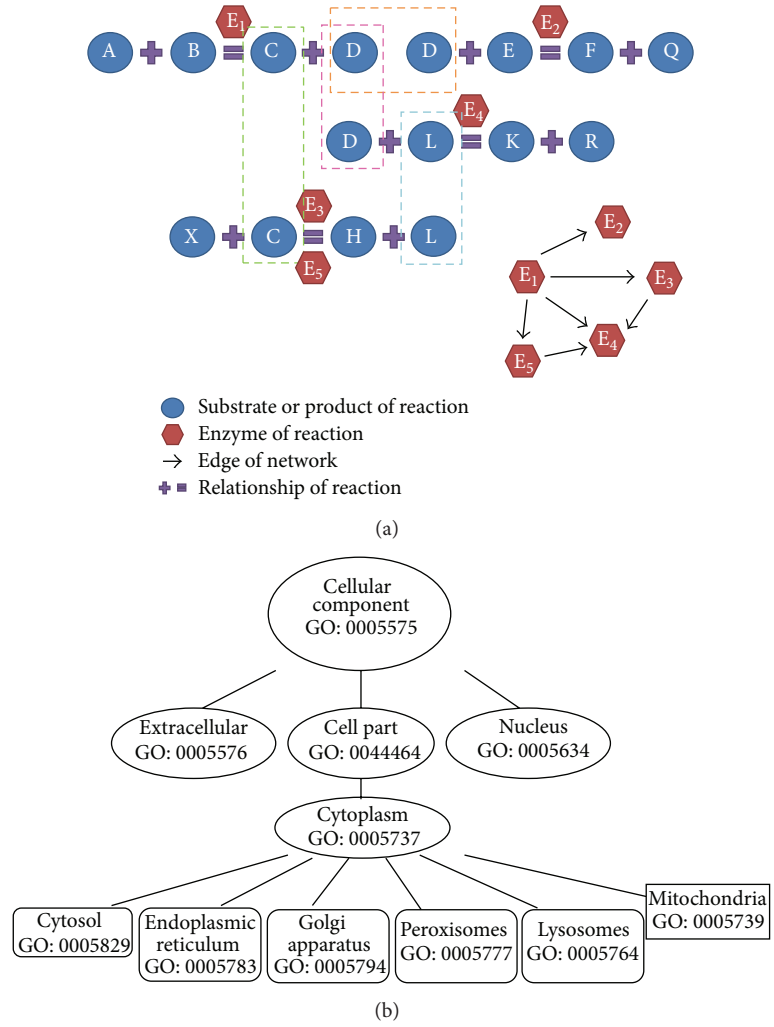

Figure 1: Schematic diagram of enzyme network construction and protein location method. (a) Substrates and products of reactions are shown as blue circles, enzymes of reactions are shown as red hexagons, relationships of reaction are shown as purple mathematical symbols, edges of network are shown as black arrows, and the arrow pointing means the order of reactions. (b) The Gene ontology terms used for protein locations. The top locations are circled. Proteins are matched to GO terms and then backtracked to the selected locations through the hierarchical structure.

and dividing by the standard deviation (σ_k) of the aggregated Z-scores derived by sampling 10000 sets of k enzymes from the EHEN:

$$Z_{\text{Enzyme}}^{\text{corrected}} = \frac{Z_{\text{Enzyme}} - \mu_k}{\sigma_k}. \quad (3)$$

(4) Corrected Z-scores were then transformed to P values by using CDF:

$$P\_\text{value}_{\text{Enzyme}} = 1 - \text{cdf}\left(Z_{\text{Enzyme}}^{\text{corrected}}\right). \quad (4)$$

(5) Reporter enzymes of the EHEN were screened with P values under a significant threshold of 0.05.

(6) The network functional modules based reporter enzymes were found by MCODE [29] (with the parameters of degree cutoff ≥ 2 and K-core ≥ 2).

(7) Gene ontology (GO) enrichment analysis was performed for each module from 6; select the modules related to programmed cell death.

(8) To repeat the above method in the 6 subnetworks, we got the reporter enzymes related network functional modules based on different subnetworks. Finally, we got reporter enzymes related network functional modules in the EHEN and six subnetworks.

2.4. Functional Programmed Cell Death-Module Analysis of the EHEN and Subnetworks. In order to analyze the relationship between cell death and CHD, we select the functional modules related to programmed cell death for further research. GO functional enrichment analysis was applied for these modules using the Functional

Annotation Tool in DAVID Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov/). FDR less than 0.05 was considered as significant.

3. Result

3.1. Enzyme Network and Subnetwork from the EHMN. Using the method described above in the network and subnetwork construction, all the human proteins coming from GO were classified into the chosen locations. By the described method, our constructed EHEN was separated into 6 subnetworks according to its localization information in our study, including cytoplasm (EHEN-C), mitochondria (EHEN-M), Golgi apparatus (EHEN-G), extracellular (EHEN-E), nucleus (EHEN-N), and endoplasmic reticulum (EHEN-R).

The input for network topological analysis to the calculation is the list of relationships of enzymes. Observing the degree distribution, characteristic path length, the connectivity, network diameter, the average and the maximum of the shortest path lengths, and clustering coefficient, the EHEN and subnetworks were found as scale-free following nearly a power law model ($f(x) = a * x^b$, $a = 29.9$, and $b = -0.46$) and had small-world properties with scale-free topology, which was a general characteristic of complex biological networks (more topological characteristics of networks are in the Supplementary Material Table S1 and Figure S1 (see Supplementary Material available online at http://dx.doi.org/10.1155/2014/475379)).

3.2. The Modules Based Reporter Enzymes in EHEN and Subnetworks. From the reporter enzyme selection algorithms in the method part, we got reporter enzymes of overall and subnetwork, respectively. The numbers of reporter enzymes varied in different networks; the EHEN-C had the largest number of reporter enzymes, while the EHEN-R had only 2 selected reporter enzymes; the overall EHEN network stayed in the middle position (7 reporter enzymes), as listed in the Supplementary Material (Table S2: the reporter enzymes of overall and subnetwork). The degree and the clustering coefficient of the reporter enzymes had significant difference ($P < 0.05$) in their respective networks. It was shown that the reporter enzymes had smaller degree and clustering coefficient in the EHEN, while they had bigger degree and clustering coefficient in subnetworks (more topological characteristics of reporter enzymes are in the Supplementary Material Table S3). It implied that the reporter enzymes selected from subnetworks played a more important part in the disease emergence and tended to form network modules.

With the help of Cytoscape and its plug-ins, we selected out modules based reporter enzymes. From module division and annotation result of the EHEN and subnetworks by using Mcode, we gained functional modules of each network. Gene ontology (GO) enrichment analysis was performed for seven modules using DAVID, including 2 modules in the EHEN, 2 modules in the EHEN-C, and 3 modules in the EHEN-M (see Figure 2, and more information of modules in the Supplementary Material Table S4).

3.3. Literature Retrieval and Functional Annotation of the Reporter Enzyme and Related Modules. CHD was caused by the plaques buildup of lipid, which was created by macrophages forming foam cells. We defined the classification principles based on CHD-related functions-CHD pathogenesis, lipid metabolism, and programmed cell death (Table 1). Most of the modules could be enriched to the basic metabolic function class, the related CHD pathogenesis, and the related lipid function class. For instance, asparagine synthase was a reporter enzyme in both the EHEN and the EHEN-M and significantly inhibited the proliferation of cells [30]. In the module EHEN-M2, another reporter enzyme ec: 1.14.15.6, cholesterol monooxygenase (CYP11A1), also known as cytochrome P-450scc was the most important family of enzymes in microsomal mixed function oxidase, widely distributed in vivo, and it was detected human cardiac expression of the mRNAs for many of the enzymes involved in the formation of adrenal corticosteroids, supporting the possibility of local production of corticosteroids and a physiological role for these hormones in cardiac function [31]. In the module EHEN-C1, the reporter enzyme ec: 6.1.1.3, threonyl-tRNA synthetase (TARS), was an autoantigen in the autoimmune disorder myositis, and borrelidin, which was a potent inhibitor of TARS, inhibits angiogenesis. TARS thus provided a potential target for detecting or interdicting disease-related inflammatory or angiogenic responses [32]. And the reporter enzyme ec: 3.1.4.37 (2′,3′-cyclic-nucleotide 3′-phosphodiesterase), which was enriched on the function classification related lipid, regulates intracellular cAMP levels, which might represent novel therapeutic agents to limit angiogenesis in complex human diseases [33].

Because coronary heart disease is caused by abnormal lipid metabolism, so the GO terms associated with lipid metabolism can be direct evidence between reporter enzymes and CHD. We were more interested in related programmed cell death reporter enzymes and modules.

3.4. Literature Retrieval and Functional Annotation of the Module Related to Programmed Cell Death. The module 3 in mitochondria subnetwork (EHEN-M3) was a module which enriched to not only the basic metabolic function class, the related CHD pathogenesis, but also the related programmed cell death function class. It included 33 enzymes (2 reporter enzymes ec: 6.1.1.7 and ec: 6.1.1.17 and 31 others) (Figure 3(a)). Through GO enrichment analysis, the genes of these enzymes in the module enriched 47 GO terms, including 15 GO terms related programmed cell death, 14 GO terms related CHD, and 18 GO terms related basic metabolic (Figure 3(b)). In this module, we were interested in the part of programmed cell death. For the reporter enzyme ec: 6.1.1.17 (glutamate—tRNA synthetase), it was proved that glutamate—tRNA synthetase of *Bacillus subtilis* was known to result in the death of the host cell [34]. For the reporter enzyme ec: 6.1.1.7 (alanyl-tRNA synthetase), it was proved that a homozygous missense mutation in AARS2 causes perinatal or infantile cardiomyopathy with near-total combined mitochondrial respiratory chain deficiency in the heart, in which AARS2 is identified to encode mitochondrial alanyl-tRNA synthetase [35].

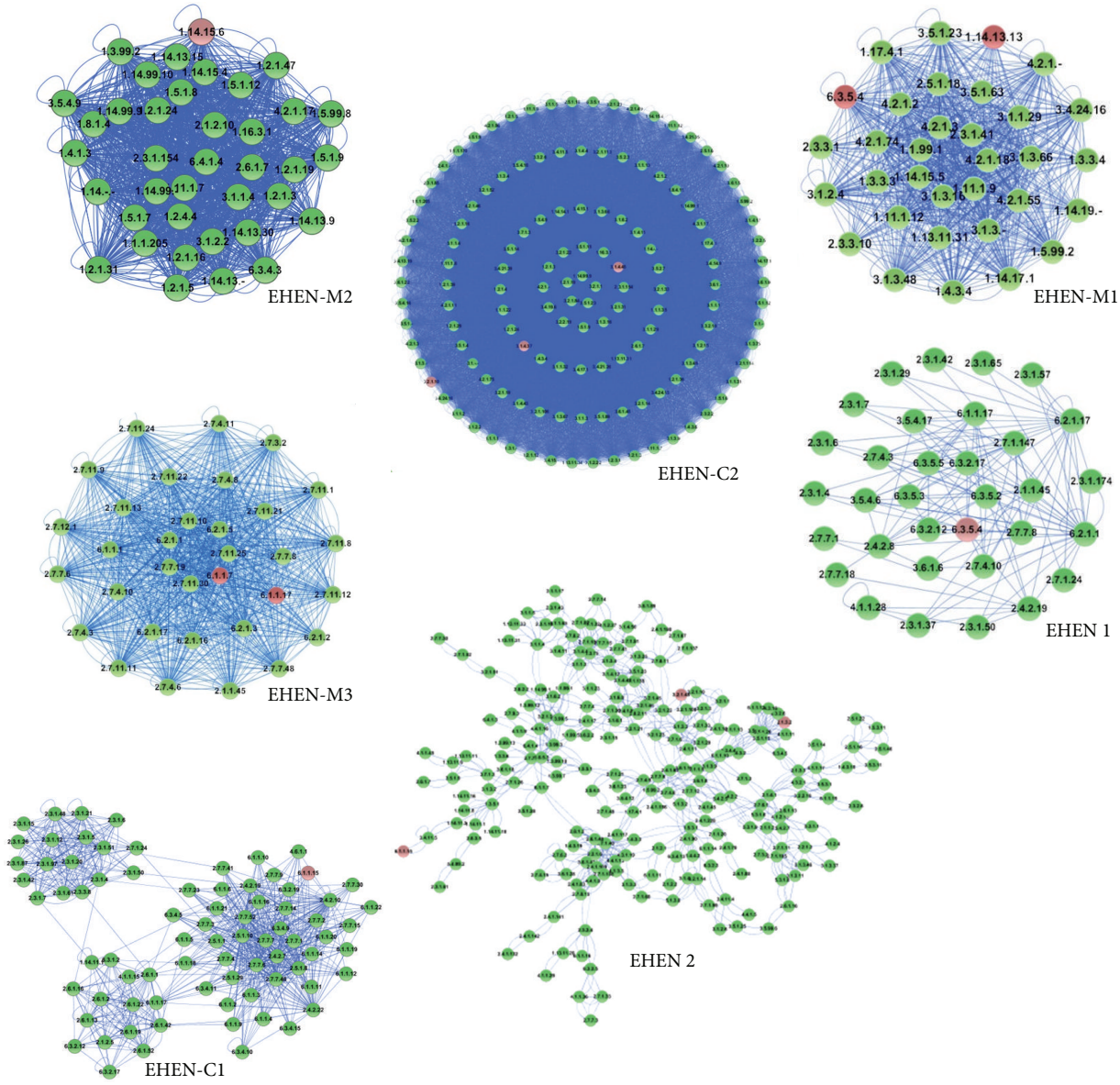

Figure 2: Seven modules based on reporter enzymes.

Table 1: Module information of overall and part of subnetworks.

Network	Module count	RE count	Related function count			
			Basic metabolic	Lipid	CHD	Death
EHEN	2	4	32	18	9	—
EHEN-C	2	6	40	22	22	—
EHEN-M	3	5	51	13	35	15

We found there were 13 enzymes enriched to GO terms related programmed cell death, and more of them belonged to serine/threonine kinases. For instance, the enzyme ec: 2.7.11.10, the inhibitor of NF-κB kinase subunit β (IKKβ), formed a transduction complex that controls the production of proinflammatory cytokines mediating cardiomyocyte hypertrophy, and activation of IKKβ in turn enhances fetal gene expression and cardiomyocyte growth [36]. The enzyme ec: 2.7.11.22 (cyclin-dependent kinase) could interact with p27 in neonatal rat cardiomyocytes, which exerted antiapoptotic and growth-inhibitory effects, and may help to improve heart function and survival in rodents [37, 38].

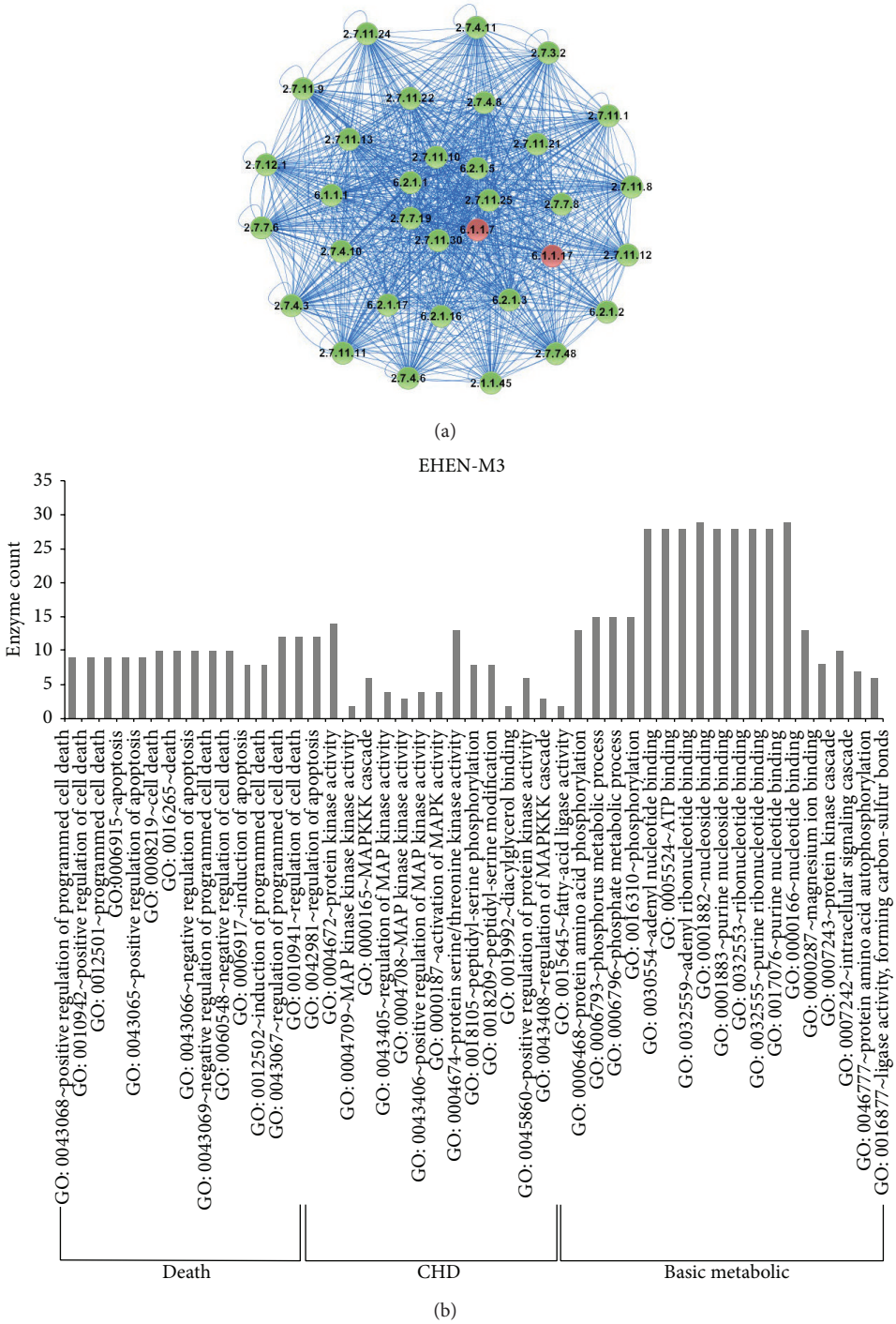

Figure 3: The module EHEN-M3 and the result of the functional enrichment. (a) The module 3 in EHEN-M. (b) The result of the functional enrichment.

4. Discussion

Traditionally, there are several ways to research the mechanism of CHD. Our approach focused on CHD in the programmed cell death perspective on the systems biology level. We constructed a correlative network of enzymes considering the cascade. The differences from the macrophages to the foam cell between disease patients and normal controls were calculated according to the expression level. We proposed a computational approach to select 7 modules based on reporter enzymes (2 in the EHEN, 2 in the EHEN-C, and 3 in the EHEN-M). Most of the modules could be enriched to the basic metabolic function class, the related CHD pathogenesis, and the related lipid function class.

The module EHEN-M3 was related to programmed cell death in the EHEN-M. Functional and structural integrity of mitochondria was essential for the physiological function of the cardiovascular system. Accumulation of mitochondrial DNA mutations has been linked to ischaemic heart disease, cardiomyopathy, and atherosclerotic vascular disease. Mitochondria are known to regulate apoptotic and autophagic pathways that have been shown to play an important role in the development of cardiomyopathy and atherosclerosis [39]. Our results also verified it from a new perspective of enzyme, which was a new research direction about CHD.

It was reported that macrophages and foam cells were associated with CHD in the aspect of programmed cell death. Wang et al. reported that macrophage with lipid growing eventually formed foam cells until death. A large pool of bubble formation and programmed cell death eventually developed into a typical plaque [40, 41]. More importantly, the reporter enzymes were obtained from macrophages and foam cells, which might be isolated from the peripheral blood of patient. Modules could be identified based on these reporter enzymes to reflect the disease state.

Deficiency or alterations in metabolic functions were known to be involved in CHD. Enzyme proteins and chemical compounds were connected in metabolic networks. The reconstruction of the enzyme network could illustrate cascade relationships of enzymes. The selected reporter enzymes and modules from each subnetwork were more closely associated with CHD. We hope that the research would be more comprehensive with more data accumulation, such as posttranslational regulatory data. Considering from this aspect, enzyme marker and modules could be searched and be used in disease detection in the future.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Xu Jia, Wan Li, and Zhengqiang Miao contributed equally to this work.

Acknowledgments

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Review Article

Cell Death and Inflammatory Bowel Diseases: Apoptosis, Necrosis, and Autophagy in the Intestinal Epithelium

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Cell death mechanisms have been associated with the development of inflammatory bowel diseases in humans and mice. Recent studies suggested that a complex crosstalk between autophagy/apoptosis, microbe sensing, and enhanced endoplasmic reticulum stress in the epithelium could play a critical role in these diseases. In addition, necroptosis, a relatively novel programmed necrosis-like pathway associated with TNF receptor activation, seems to be also present in the pathogenesis of Crohn's disease and in specific animal models for intestinal inflammation. This review attempts to cover new data related to cell death mechanisms and inflammatory bowel diseases.

1. Introduction

1.1. Cell Death and Damage Control. The inflammatory process aims to neutralize harmful stimuli as an effort of self-protection [1]. There are basically two types of inflammation: acute and chronic. Acute inflammation comprises the initial response to eliminate the insulting cause without any residual structural or functional damage. It is a temporary phenomenon, which includes later regeneration and complete healing of the involved area [1]. In contrast, when the initial insult persists, the resulting chronic inflammation leads to organ damage, preventing a complete return to homeostasis [2]. In the inflamed gut, both in acute and in chronic inflammation, an effective modulation of the immune response with the subsequent downregulation of inflammation is critical to reduce tissue damage and to promote mucosal healing [3]. In this sense, the programmed cell death machinery is key for the homeostasis reestablishment after an acute or chronic insult, limiting the propagation of the inflammatory stimuli to prevent tissue's loss of function [4].

In vitro studies have demonstrated resistance to apoptosis in lamina propria T cells obtained from the intestinal mucosa of patients with Crohn's disease (CD) [6]. Additional evidence has long supported the association of T cell resistance to apoptosis with altered concentration ratios of Bcl-2 family proteins [7, 8]. In fact, efficacy of anti-TNF-alpha antibodies in inflammatory bowel diseases (IBD) has been associated with apoptosis modulation in lamina propria mononuclear cells, in particular T cells [9, 10], through the induction of the intrinsic apoptotic pathway mediated by Bcl-2 family proteins [11]. Recently, the defective apoptosis of lamina propria T cells in CD was also shown to be related to increased levels of survivin, a family member of the inhibitor of apoptosis proteins (IAP), through the interaction with the chaperone HSP90 [12]. Nevertheless, in the last decade, research in IBD pathogenesis has undergone a progressive shift from the effector arm of inflammation, namely, the adaptive immune system, towards the innate immunity and mechanisms involving the complex interactions between the host and the microbiota.

In recent years, several genome-wide association studies (GWAS) have been undertaken in IBD patients and healthy controls providing an extraordinary new insight into the pathogenesis of these conditions [13–15]. The combined

genome-wide analysis of CD and ulcerative colitis (UC) generated a more comprehensive analysis of disease specificity [16]. Currently, the total disease variance explained by heritability in IBD ranges from 7.5% in UC to 13.6% in CD with 110 of 163 loci associated with IBD being found in both diseases [16]. Most known susceptibility genes are involved in autophagy, cellular stress regulation, and microbial pathogen sensing, suggesting that cell death mechanisms might play a key role in the pathogenesis of IBD.

1.2. Homeostasis of Intestinal Epithelium. The intestinal epithelium constitutes a specialized single cell layer with absorptive and secretory functions in the interface between the body and the external environment [17]. In the epithelium, enterocytes are responsible for the absorption of nutrients, ions, vitamins, and water and are also involved in the induction of immunological tolerance to ingested peptides [18]. Paneth cells, goblet cells, and enteroendocrine cells comprise the secretory lineage of the intestinal epithelium, having an important role in the intestinal defense against potentially harmful bacteria and the coordination of intestinal functions by hormone secretion [19–21]. In close contact with the epithelium lies the lamina propria, a loose connective tissue in which mesenchymal cells and mucosal immune cells are located.

In the large and small intestine, differentiated enterocytes are removed constantly and replaced by new cells originated by undifferentiated adult intestinal stem cells, localized in the third or fourth position counted from the base of the crypt [22]. These new cells migrate from the base of the crypt to the apical zone of the intestine undergoing maturation. In the apical zone, these cells survive for about 4-5 days prior to being shed into the gut lumen [23]. This single epithelial layer displays a strict balance between cellular proliferation and cell death in order to maintain the intestinal barrier [24]. Importantly, if the epithelium cell death is not strictly regulated, it might result in a barrier defect with subsequent microbial invasion and inflammation. In this regard, previous studies have shown that epithelial proliferation and turnover are accelerated in IBD, with elevated levels of programmed cell death being observed in patients with both CD and UC [25, 26].

In IBD, all three types of programmed cell death are observed: apoptosis, autophagy, and necrosis (Figure 1). The exact programmed cell death pathway a cell undergoes depends on several factors such as the abundance of nutrients, the cell cycle stage, and the presence or absence of reactive oxygen species (ROS), adenosine triphosphate (ATP), autophagy protein 5 (ATG5), and nuclear factor kappa B (NFκB) activation, among others [27–31].

2. Apoptosis

2.1. Intracellular Machinery of Apoptosis. Even though caspase-independent mechanisms mediated by the apoptosis-inducing factor (AIF) have been described, the activation of caspases is classically required to initiate the process of apoptosis [32]. Caspases comprise a specialized protease family, which contains a cysteine on the active site that cleaves the targets on their specific aspartic acid. Caspases not only participate in the progressive activation of other caspases but can also contribute to other processes such as the reduction of cell volume (pyknosis), chromatin condensation, nuclear fragmentation (karyorrhexis), and formation of plasma-membrane blebs [33, 34]. All these processes lead to alterations in cellular morphology resulting in cell and nucleus shrinkage without leakage of cellular content to the microenvironment. The intracellular machinery of apoptosis involves extrinsic and intrinsic pathways.

The extrinsic pathway also known as death receptor pathway involves the activation of death receptors, which are triggered by APO3L, TNF-α, FAS-L, and TNF-related apoptosis-inducing ligand (TRAIL). These ligands bind to their specific receptors such as APO3, TNF receptor (TNFR), FAS, and DR4/DR5 [3]. The ligand-receptor interaction initiates the destruction complex through the recruitment of intracellular adapted proteins called Fas associated with death domain (FADD) or TNF-α receptor-associated death domain (TRADD) that enables the catalytic activity of caspase-8, the central protease mediator of the extrinsic pathway [35].

The intrinsic pathway is observed when cells are under conditions such as DNA damage or growth factors withdrawal. In case of failure to repair the subsequent damage, the intracellular machinery stimulates the transcription of p53 [36, 37]. This gene, known as the guardian of the genome, stimulates other proteins such as p53 upregulated modulator of apoptosis (PUMA), Bcl-2 interacting mediator of cell death (BIM), and NOXA to initiate the cell death cascade [38–40]. The family of proteins that control the intrinsic pathway is known as Bcl-2. This family includes antiapoptotic and proapoptotic members. The difference between them lies in their homologous domains. The antiapoptotic members have four Bcl-2 homology regions and the proapoptotic members have three [41]. In addition, there is a third class of proapoptotic Bcl-2 family members that displays only the Bcl-2 homology 3 domain ("BH3-only") [42].

In the intrinsic pathway, the balance between antiapoptotic and proapoptotic members is responsible for the determination of either cell death or cell recovery. When proapoptotic stimuli are prevalent, t-BID interacts with BAK and BAX leading to increased mitochondrial permeability and release of electron carrier protein cytochrome-c and SMAC/DIABLO. This protein inhibits the IAP, which are characterized by the blockage of caspase activity, while interacting with apoptotic protease activating factor 1 (Apaf-1) enabling the catalytic activity of caspase-9, the central protease mediator of the intrinsic pathway. Owing to the critical participation of mitochondria, this mechanism is also known as the mitochondrial pathway [35].

The activation of extrinsic (mediated by caspase-8) and intrinsic (mediated by caspase-9) pathways leads to activation of caspase-3, caspase-6, and caspase-7, which favors the cleavage of other proteins. A point of no return is achieved

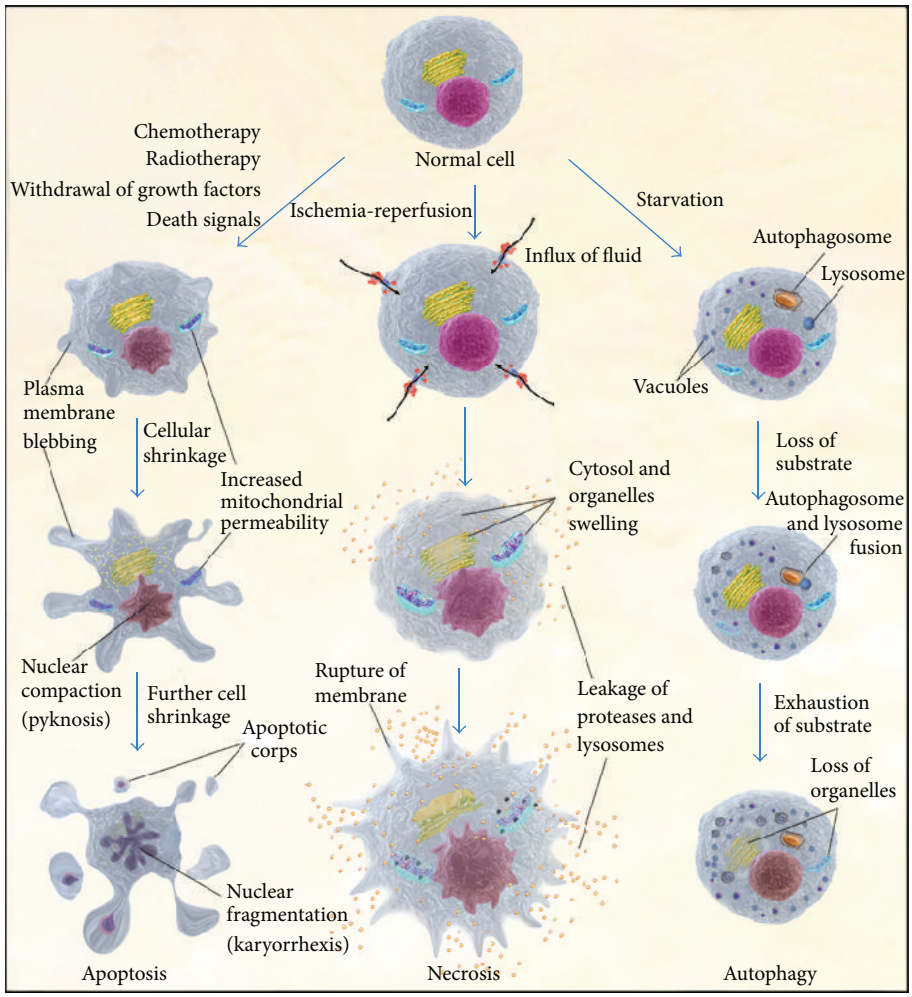

FIGURE 1: The three major pathways of cell death. Cells can be directed to different programmed cell death mechanisms depending on several factors. In the left, the apoptosis pathway is represented with the characteristic cellular shrinkage and formation of the apoptotic bodies without leakage of contents. In the middle, the necrotic pathway shows the cytosol and organelle swelling and rupture of plasma membrane with subsequent leakage of cellular contents. In the right, autophagy is illustrated with the appearance of vacuoles, the autophagosome, and its fusion with the lysosome, which ends in organelle digestion.

once the cell advances towards a critical state of destruction that will end in cell death and give rise to structures called apoptotic bodies.

2.2. Apoptosis in IBD. In the normal small intestine epithelium, all cells but Paneth cells and intestinal stem cells migrate from the base of the crypt to the villus tip where they are shed into the lumen. Bullen et al. studied almost 15.000 villus sections to closely determine the exact mechanisms behind cell shedding in the small intestine [43]. In this study, apoptotic cells were identified using antibodies against cleaved cytokeratin 18 and caspase-3. The authors found that cells always underwent apoptosis before shedding and that apoptotic bodies were never found in the epithelial monolayer. Interestingly, Marchiando et al. observed that morphologic changes typical of apoptosis were not apparent until the nucleus of the shedding cell had moved above the nuclei of adjacent cells, suggesting that, in the order of events, shedding leads to apoptosis [44]. The authors also demonstrated cleaved caspase-3 staining within the cytoplasm of shedding cells, which was only detectable after cell shedding was evident [44]. A broad-spectrum caspase inhibitor was then used and it was shown that almost all shedding events were blocked, indicating that caspase-3 cleavage is critical for cell shedding to occur [44].

In contrast, it has been shown that mice lacking caspase-3 (and caspase-8 and FADD as well) display limited apoptotic phenotype with no impact on gastrointestinal homeostasis [45–48]. In this regard, the activity of caspase-independent cell death pathways in the gut might be an important safeguard when caspase-mediated routes fail [32]. Interestingly, the early event in the cell shedding process seems to be the reorganization of ZO-1 and occludin which is accompanied by partial microvillus vesiculation and intracellular organelle breakdown, progressing to complete vesiculation of microvilli, nuclear condensation, and terminal contraction of surrounding epithelia [44].

Different from intestinal cell shedding, patterns of spontaneous apoptosis in the small and large intestine were more extensively described mostly due to the enterprise of the late Professor Christopher S. Potten [49]. Spontaneous apoptotic cells are restricted to the stem cell region in the small intestine and are seldom found in colonic crypts, being distributed along the length of the crypt [50]. This spontaneous apoptosis, which is p53-independent, has been seen as part of the stem cell homeostasis [49]. In contrast, Bcl-2 is minimally expressed in the small intestine, being more strongly expressed at the base of colonic crypts [50]. Interestingly, differences in Bcl-2 expression and cell death regulation can be accounted for the variability in tumor prevalence between the small and large intestines [50].

In IBD, high levels of apoptosis have been observed in the intestinal epithelium of patients. Our group investigated apoptosis in distinct mucosal compartments and the expression of Fas/Fas ligand in the inflamed and noninflamed intestinal mucosa of patients with IBD [51]. Colon specimens from patients with UC and CD were analyzed for densities and distribution of apoptotic cells determined by TUNEL essay. Colonic epithelium from patients with UC showed higher rates of apoptosis than controls, with no differences regarding CD [51]. Iwamoto et al. also found that apoptotic features were found in crypts of active UC, suggesting that loss of epithelial cells occurs mainly by apoptosis in involved intestine and also in adjacent uninvolved areas [52]. In keeping with these findings, Hagiwara et al. observed that the apoptotic indices in UC patients were significantly higher than those in controls but similar to those in infectious colitis patients [53]. Interestingly, apoptotic indices were significantly higher in patients undergoing surgery compared to those on medical treatment perhaps due to different disease severities [53].

In proteomic analysis, data also point towards the association between apoptosis and IBD. In this regard, in a small intestinal epithelial cell proteome study comparing CD, UC, and controls, 47% of all changes in the epithelial cell proteome were associated with signal transduction pathways, which included proapoptotic mechanisms [54]. In this study, the programmed cell death protein 8 (PDCD8) associated with caspase-independent apoptosis was almost 8-fold upregulated in inflamed versus noninflamed tissue regions in UC patients, supporting that programmed cell death mechanisms contribute to conditions of chronic inflammation in the gut [54]. As UC is mostly associated with a T helper type 2 (Th2) immune response, studies have suggested that Th2 cytokines might play a role in the enhanced apoptotic ratio found in the intestinal epithelium of patients with UC. In this regard, Rosen et al. observed that increased STAT6-dependent levels of IL-13 in UC were associated with greater epithelial cell apoptosis and barrier dysfunction and suggested that inhibition of STAT6 might decrease apoptosis in the epithelium of new-onset ulcerative colitis [55]. In accordance with these findings, IL-13 had a dose-dependent effect on transepithelial resistance of HT-29/B6 monolayers due to an increased number of apoptotic cells with parallel changes being observed in human samples [56].

Several animal studies further confirm the central role of apoptosis in disease mechanisms in IBD. The knockout mice for XBP1 (an endoplasmic reticulum (ER) stress-related transcription factor), for instance, develop spontaneous enteritis and are associated with Paneth cell dysfunction and subsequent apoptotic cell death [57]. More importantly, in humans, an association between UC and CD with XBP1 variants was identified and replicated as susceptibility genetic factors [57]. Likewise, NF-kappa B deficiency was shown to lead to apoptosis of colonic epithelial cells with subsequent impaired expression of antimicrobial peptides and translocation of bacteria into the mucosa [58, 59]. Another example is the conditional STAT3 knockout mice in intestinal epithelial cells; these animals were found to be highly susceptible to experimental colitis with important defects in epithelial restitution and enhanced apoptosis [60]. It has been further suggested that luminal nutrients and the microbiota can also influence the apoptotic ratio in the intestinal epithelium in mice. In this regard, luminal iron was shown to trigger epithelial cell stress-associated apoptosis through changes in microbial homeostasis [61]. In this study, in a CD-like ileitis mouse model, mice developed severe inflammation of the distal ileum with enhanced expression of proapoptotic cleaved caspase-3. Interestingly, absence of luminal iron sulfate reduced the expression of cleaved caspase-3 in the ileal epithelium [61].

In CD, the percentage of apoptotic enterocytes was found to be higher in involved compared to uninvolved areas and normal intestine, with no significant difference being found between uninvolved and normal mucosa [25]. These findings suggest that a greater apoptosis ratio in the intestinal epithelium of CD is associated with intestinal inflammation, being exclusively increased in inflamed areas [25]. Apoptosis was also observed after infection with several intestinal pathogens including *Salmonella*, *Shigella*, enteropathogenic *Escherichia coli*, human immunodeficiency virus type 1, *Helicobacter pylori*, and *Cryptosporidium parvum* [62]. In the case of infectious involvement of the intestine, pyroptosis, another form of cell death similar to apoptosis but less characterized, was also observed [63, 64]. This type of cell death forms a complex of proteins called inflammasome (or pyroptosome) that requires caspase-1 and activates interleukin-1 beta (IL-1β) and IL-18, two types of proinflammatory cytokines, which are predominant in T helper cells type 1 (Th1) immune responses [65].

In the gut, inflammasome activation has been largely associated with the nucleotide-binding-oligomerization-domain- (NOD-) like receptors, which can sense bacterial components and also noninfectious elements regarded as damage-associated molecular pattern (DAMPS), molecules that can initiate and perpetuate immune response (Figure 2) [66]. In particular, NLRP3 is a NOD-like receptor that can be triggered by bacterial constituents and also by synthetic purine-like compounds, endogenous urate crystals, and exogenous adenosine triphosphate (ATP) [67]. Furthermore, it has been postulated that NLRP3 inflammasome activation can be mediated by pannexin-1 and $P2X_7$ receptor, a member of the ATP-activated P2X purinergic receptors family [68]. The $P2X_7$ receptors have been shown to function as danger

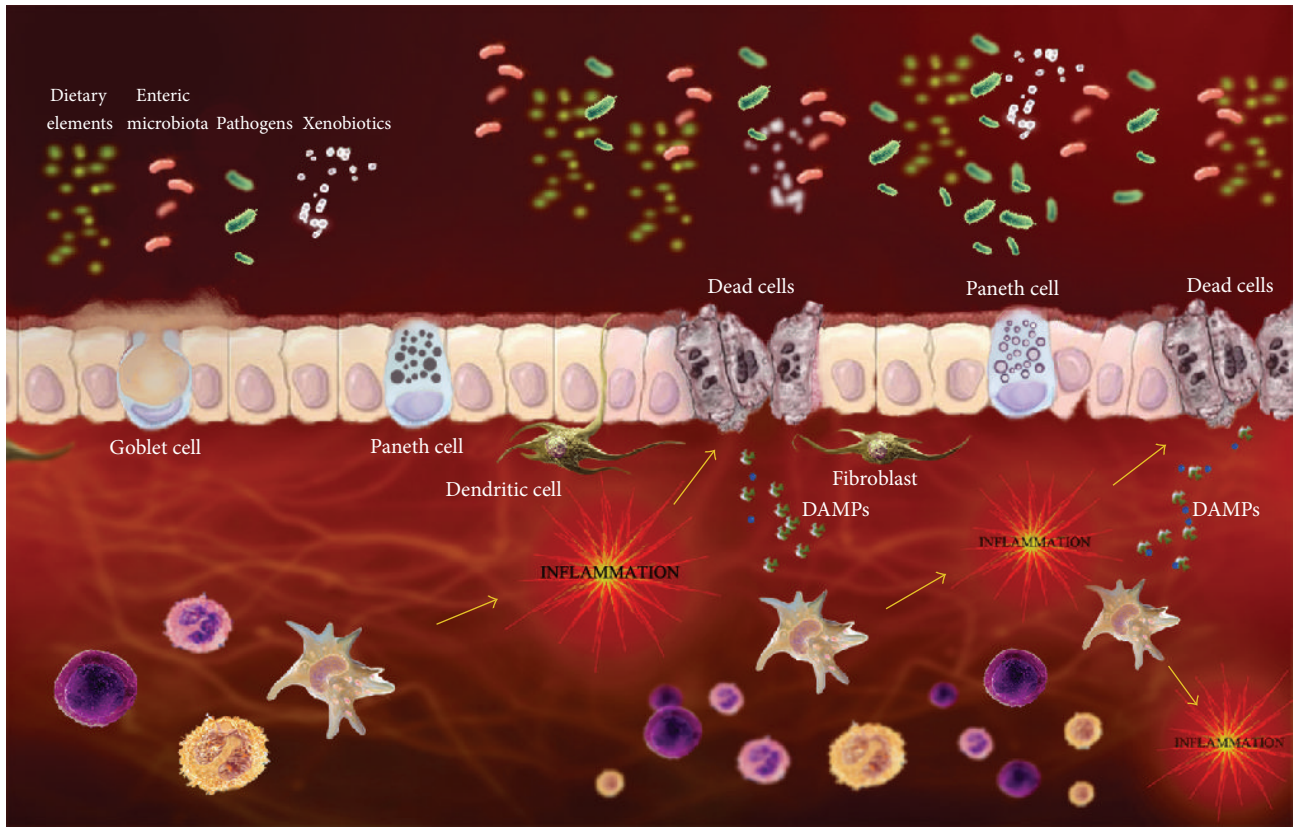

Figure 2: Simplified cartoon of the integrated intestinal homeostatic mechanisms showing the interplay between cell death and innate immunity in intestinal inflammation. Abnormal bacterial sensing through NOD-like and TLR in epithelial cells and dendritic cells in addition to Paneth cell dysfunction are greatly interrelated with the unfolded protein and autophagy pathways. The resulting production of chemokines and cytokines and the activation of immune cells in the lamina propria determine further epithelial barrier defects, with additional exposure to diverse intraluminal contents, enhanced by contact with damage-associated molecular patterns (DAMPs), in a self-perpetuating amplification loop. Figure adapted from Nunes et al. [5].

sensors in immune cells and have been implicated in different biological functions, including apoptosis and the production and release of proinflammatory cytokines [69]. In addition, ATP was shown to induce apoptosis and autophagy in human epithelial cells, possibly via reactive oxygen species production [27]. These data, in conjunction with recent results from our group comprising experimental colitis [29] and human IBD [28], support the involvement of $P2X_7$ receptors and the consequent inflammasome activation in the pathogenesis of IBD.

When it comes to response to therapy, polymorphisms in apoptosis genes were found to predict response to anti-TNF therapy in luminal and fistulizing CD [70]. In a cohort of 287 consecutive patients treated with infliximab, Fas ligand and caspase-9 genotypes predicted the outcomes after anti-TNF therapy. Interestingly, concomitant thiopurine therapy overcame the effect of unfavorable genotypes [70]. Regarding the effects of anti-TNF therapy on epithelial cells apoptosis, Zeissig et al. showed that, after anti-TNF treatment, a down-regulation of epithelial apoptosis takes place in active CD [71]. In this study, the epithelial apoptotic ratio was increased in CD compared to controls and subsequently decreased after anti-TNF was introduced [71]. Marini et al. observed that anti-TNF therapy decreases the severity of murine CD-like ileitis by abolition of intestinal epithelial cell apoptosis [72]. In this study, a single injection of anti-TNF resulted in a marked suppression of intestinal inflammation, with a significant reduction in epithelial apoptosis. In contrast, an increase in lamina propria mononuclear cell apoptosis was observed. These results were confirmed in vivo by TUNEL staining, demonstrating that anti-TNF therapy involves homeostatic regulation of mucosal cell apoptosis [72].

3. Necrosis

3.1. Intracellular Machinery of Necrosis. Necrosis is derived from the Greek word "nekros" and means corpse [73]. To initiate the process of necrosis, the store of ATP is depleted by PARP (an enzyme which participates in DNA repair), which determines the shift from apoptotic to necrosis [74]. In the necrotic process, cell and organelles swell and rupture with subsequent leakage of cellular content to the microenvironment causing an inflammatory response. Until recently, cells

were believed to passively undergo necrosis after external environment changes such as intestinal ischemia, inflammation, significant alterations in temperature, pH, and mechanical force [31, 75–77].

In the last two decades, however, several groups demonstrated that cells could undergo a necrosis-like cell death after TNF incubation [78–80]. Additional work described that this particular form of programmed cell death was triggered by death receptors and stimulated by caspase-8 inhibition [81, 82]. Because of its fine regulation, this cell death mechanism was posteriorly called necroptosis or programmed necrosis [83]. Necroptosis is characterized by the same morphologic features of necrosis as cell swelling, mitochondria dysfunction, membrane permeabilization, and release of cytoplasmic content, being also associated with high mitochondrial reactive oxygen species (ROS) production and it does not involve DNA fragmentation [84].

Necroptosis can be activated by lipopolysaccharides (LPS), physical-chemical stress, ionizing radiation, calcium overload, anticancer drugs, and DNA damage among other stimuli [84]. Signaling can be initiated through activation of members of the tumor necrosis factor (TNF) family and this pathway has been shown to be mediated by kinases receptor-interacting protein 1 (RIP1) and receptor-interacting protein 3 (RIP3) [47]. Upon induction of necrosis, RIP3 is recruited to RIP1 to establish a necroptosis inducing protein complex [47].

3.2. Necrosis in IBD. Cytotoxic bacteria were shown to induce necrosis in intestinal epithelial cells, which indicates that this cellular death process has an important role in infectious gastrointestinal diseases [85]. In CD, necrosis had been observed in electron and light microscopic of the intestinal epithelium [86]. In this study, samples from patients with CD, UC, and controls were evaluated. Patchy necrosis without acute inflammation was observed exclusively in patients with CD, indicating that this finding could have developed prior to inflammation [86].

Recently, two independent groups assessed the role of the programmed necrosis in IBD. Christoph Becker's group demonstrated the role of Caspase-8 in the regulation of necroptosis in the intestinal epithelium [47]. In this study, mice with a conditional deletion of caspase-8 in the intestinal epithelium spontaneously developed terminal ileitis and were highly susceptible to DSS colitis [47]. These mice also lacked Paneth cells, indicating dysregulated antimicrobial immune cell functions in the intestinal epithelium. In addition, epithelial cell death was induced by TNF-α and was associated with increased expression of RIP3 [47]. More importantly, the authors identified high levels of RIP3 in human Paneth cells and increased necroptosis in the terminal ileum of patients with CD, suggesting a potential role of necroptosis in the pathogenesis of this disease. In the other study, Welz et al. showed that knockout mice for FADD in intestinal epithelial cells spontaneously develop epithelial cell necrosis with loss of Paneth cells and small and large bowel inflammation [48]. In addition, MYD88 deficiency or elimination of microbiota prevented colon inflammation, indicating that toll-like receptor signaling drives the pathology in these animals [48].

4. Autophagy

4.1. Intracellular Machinery of Autophagy. Autophagy is derived from the Greek word that means "self-eating" [87]. This process is mainly known as the cell mechanism to recycle its own nonessential organelles, which can be activated by the lack of nutrients and growth factors in the extracellular microenvironment [88]. The characteristic structures of autophagy are the vacuoles, slight chromatin condensation, and the autophagosome, which fuses with lysosomes to digest material into substrates [87, 89]. The autophagosome is best visualized by electron microscopy and is composed of a double membrane lysosomal-derived vesicle that catabolizes the nonessentials or damaged particles and organelles [90]. The intracellular machinery of autophagy is composed of a complex of proteins formed by the class III phosphatidylinositol-3-kinase (PI3K), also known as Vps34, and the Bcl-2 interacting BH3 domain protein, Beclin-1 (BECN1). Both proteins are required for the autophagosome formation [91]. Signaling can be initiated through the mammalian target of rapamycin (mTOR) pathway, a serine/threonine kinase that participates in several mechanisms involved in cell survival.

Autophagy constitutes a self-degradation process, representing a critical mechanism for cytoprotection of eukaryotic cells. However, in the context of cancer, autophagy appears to play an ambiguous role. In association with apoptosis, autophagy can act as a tumor suppressor. On the other hand, defects in autophagy, in concert with abnormal apoptosis, may trigger tumorigenesis and also therapeutic resistance [92, 93].

4.2. Autophagy in IBD

4.2.1. ATG16L1. A link between IBD and autophagy was first established when an association between CD and a single-nucleotide polymorphism (SNP) in the autophagy-related 16-like 1 gene (ATG16L1) was first reported by Hampe et al. [94] and later replicated by the same group [95]. This SNP (rs2241880) resulted in a threonine-to-alanine substitution at the amino acid position 300 of the protein (T300A) [94]. ATG16L1 is a central adaptor in the autophagosome formation. The rs2241880 variant is commonly found in the population, with 45–50% of healthy subjects carrying the polymorphism [96].

In the first study by Hampe et al., using haplotype and regression analysis, the authors found that the rs2241880 SNP carried all disease risk exerted by the ATG16L1 locus associated with CD in 3 European cohorts of CD patients [94]. Importantly, this association was not observed in a German cohort of UC cases, suggesting that the underlying biological process was specific to CD [94]. In their second study, the authors found that only individuals who were homozygous for the T300A-encoding variant of ATG16L1 were under higher risk to develop CD, suggesting a recessive model for the action of ATG16L1 [95]. In addition, a higher frequency of the rs2241880 allele was found in patients with ileum involvement, being the association with small bowel disease still significant even after adjustment for CARD15/NOD2 mutations [95]. This association with ileal involvement was

confirmed by some [97] and could not be replicated by others [98]. A highest frequency of the rs2241880 SNP was also observed in individuals with childhood-onset CD [95] but others argue that these differences are driven by variations in disease location between late- and early-onset CD [97].

After the association between ATG16L1 polymorphisms with the development of CD was established, efforts were made to determine disease-related mechanisms, which could explain this specific susceptibility. Saitoh et al. generated ATG16L1 mutant mice and examined its function in autophagosome formation and the regulation of immune responses [99]. ATG16L1 mutant mice expressed deleted forms of the protein lacking the entire coiled-coil domain [99]. Most ATG16L1-deficient mice died within 1 day, indicating that the protein was required for neonatal survival [99]. In addition, in mouse embryonic fibroblasts (MEF) from ATG16L1-deficient mice, formation of autophagosomes under starved conditions was not observed, suggesting that ATG16L1 was essentially required for autophagy [99]. Furthermore, the authors examined the impact of ATG16L1 on cytokine production in response to lipopolysaccharide (LPS), showing that IL-1β and IL-18 were highly upregulated in ATG16L1-deficient cells compared with wild-type after toll-like receptor stimulus [99]. Cleaved caspase-1, an activated form that mediates processing of IL-1β, IL-18, and apoptosis, was also detected in the supernatants of ATG16L1-deficient macrophages following LPS stimulation. Importantly, these results indicated that toll-like receptor signaling is only associated with the formation of autophagosomes in nutrient-deprived macrophages [99]. In vivo, Saitoh et al. also observed that ATG16L1-deficiency exacerbates inflammation in DSS-induced colitis [99]. Chimeric mice with ATG16L1-deficient hematopoietic cells died due to acute weight loss and severe inflammation in the distal colon [99]. In these mice, serum levels of the proinflammatory cytokines IL-1β and IL-18 were significantly elevated and their mortality rate was improved after injection of neutralizing antibodies for these cytokines, indicating that autophagy might play a protective role in massive inflammation during acute colitis [99].

Cadwell et al. were the first to show that the ATFG16L1 protein was critical for the biology of Paneth cells [100]. In mice, ATG16L1- and ATG5-deficient Paneth cells exhibited notable abnormalities in the exocytosis pathway. In addition, ATG16L1-deficient Paneth cells had increased expression of genes involved in the lipid metabolism of acute phase reactants and adipocytokines [100]. In addition, CD patients who were homozygous for the ATG16L1 risk allele displayed Paneth cell abnormalities similar to those observed in ATFG16L1-deficient mice and expressed also increased levels of leptin [100]. Later, the same group also showed that ATFG16L1 deficiency alone was not enough for the development of Paneth cell abnormalities [101]. In this regard, mice housed at an enhanced barrier facility were similar to wild-type controls, failing to display the aberrant phenotype [101]. These results suggest that Paneth cell abnormalities associated with ATFG16L1 deficiency require an exogenous factor displayed in the microbiota of mice sitting at conventional animal facilities [101]. In the intestine, further studies also suggested that defects in the ATFG16L1 autophagy pathway are important in the presence of bacteria. Cooney et al. observed that NOD2 triggering induces autophagy in dendritic cells, which required ATG16L1, and that NOD2-mediated autophagy was necessary for CD4+ T cell responses in dendritic cells [102]. The relationship between NOD2 and ATG16L1 is not solely related to autophagy. Sorbara et al. have shown that knockdown of ATG16L1 expression specifically enhances NOD-driven cytokine production and that these findings also occurred in cells with an autophagy-incompetent truncated form of ATG16L1 [103].

Others also suggested that the impact of the ATG16L1 risk allele on CD might not be exclusively related to abnormalities in autophagy. Fujita et al., for instance, have shown that the T300A mutant has little impact on autophagy against *Salmonella*, proposing that this variant is differentially involved in CD and canonical autophagy [104]. In keeping with these findings, Messer el al. found that ATG16L1-deficient cells were resistant to cellular invasion by *Salmonella* [105]. Conway et al., however, demonstrated that autophagy was induced in small intestine and cecum of mice after Salmonella infection and this required ATG16L1 [106]. In this study, *Salmonella* colocalized with microtubule-associated protein 1 light chain 3β in the intestinal epithelium of control mice but not in mice lacking ATG16L1 in epithelial cells [106]. Consistent with these findings, these transgenic mice had increased inflammation and systemic translocation of bacteria compared with control animals. In this regard, autophagy is important for the maintenance of cellular homeostasis after infection, participating in the clearance of pathogens found in the ileum of CD patients [107].

Murthy et al. filled the gaps between autophagy, apoptosis, and inflammation, suggesting that the T300A variant causes sensitization to caspase-3-mediated cleavage of ATF16L1 [108]. The authors demonstrated that caspase-3 activation leads to accelerated degradation of ATG16L1 in the presence of the T300A variant. They propose that, in healthy intestine, the turnover of ATG16L1 is dependent on basal caspase-3 activity; in the presence of T300A, however, the persistence of apoptotic stimuli enhances ATG16L1 cleavage, triggering cytokine production and inflammation [108]. More recently, an association between autophagy and the ER stress response gene Xbp1 was shown to synergistically prevent ileal inflammation [109]. In this regard, Arthur Kaser's group has shown that Xbp1 loss in intestinal epithelial cells induced autophagy, most notably in Paneth cells, as a compensatory mechanism in intestinal epithelial cells upon sustained ER stress [109]. Mice with impaired ER stress signaling and autophagy developed transmural inflammation, characterized by acute and chronic inflammation extending to the muscularis propria and serosa, as fistulizing CD. This phenotype displays the important role of autophagy in the defense against ER stress in the intestinal epithelium [109]. This model is in keeping with recent data showing that ATG16L1 T300A polymorphisms define a specific subtype of patients with CD, characterized by Paneth cell ER stress even during quiescent disease [110].

4.2.2. IRGM. Genome-wide association studies identified the autophagy gene IRGM on chromosome 5q33.1 to be strongly

associated with CD [16] and to a lesser extent with UC [15, 111]. The IRGM gene belongs to immunity-related GTPases, a family of genes in mammalian species induced by interferons, though the human form seems to lack interferon-responsive elements [112, 113]. Two polymorphisms of IRGM have been strongly associated with CD, a silent tag-SNP variation within the coding region (c.313C>T) and a 20 kb deletion upstream of the IRGM gene [113–116]. In this regard, the coding-sequence variation was not thought to be the source of this association due to the absence of changes in IRGM protein structure [114, 115].

Brest et al. subsequently suggested that this synonymous variant (c.313C>T) was responsible for a disruption in a miRNA-binding site in individuals with the risk haplotype (T), resulting in lack of miRNA regulation in these patients [117]. In this regard, in subjects with CD, colonic epithelial cells have striking decreased IRGM levels only in patients homozygous for the protective IRGM haplotype (C), being the expression more reduced in inflamed tissue compared to involved mucosa in remission [117, 118]. These data suggest that lack of miRNA regulation and consequent overexpression of IRGM secondary to the risk allele (T) contribute to the association of this region with CD [118]. Importantly, overexpression of IRGM was associated with lower autophagy efficacy [117]. The other polymorphism, the 20 kb deletion upstream of IRGM, was first identified by McCarroll et al. in perfect linkage disequilibrium with the most strongly CD-associated SNP causing IRGM to segregate in a risk sequence (deletion present) and a protective sequence (deletion not present) [114]. Functionally, in this study, cells lacking IRGM have decreased proportion of internalized bacteria by autophagosome and overexpression of this molecule causes an increase in autophagy activity [114]. In summary, the current evidence suggests that differences in miRNA regulation or presence/absence of the upstream deletion sequence can affect IRGM expression leading to autophagy dysfunction.

Several studies tried to correlate variants in the IRGM gene with specific CD clinical features. In this regard, a large German study assessed the influence of the IRGM SNPs on disease phenotype, also evaluating interactions with other IBD susceptibility genes, particularly ATG16L1 [119]. In this study, based on the Montreal classification of IBD, none of the IRGM SNPs investigated were associated with specific disease features in CD or UC. In contrast, other studies found some associations between IRGM SNPs and clinical outcomes. Accordingly, IRGM SNPs were associated with fistulizing CD and perianal fistulas in a large cohort of Italian patients [120], with ileal involvement in subjects in New Zealand [121] and in Portugal [122] and with ileocolonic resection in a small cohort of American patients [123]. In addition, the IRGM CD risk variant was also associated with increased antiflagellin seropositivity [124] and a positive response to biologic therapy [122].

5. Conclusion

The representation of the different cell death pathways as individual and isolated mechanisms is entirely schematic and it does not reflect reality. A large and growing body of evidence has demonstrated that there is a dynamic crosstalk and much redundancy among different types of cell death mechanisms [125–127]. In this regard, it has been shown, for instance, that TNF-α treatment can induce either apoptosis or necrosis depending on the targeted cell type, environmental conditions, and magnitude of the cellular insult [125]. In addition, the death receptors FAS, TNFR2, TRAILR1, and TRAILR2, which are characteristically associated with apoptosis, might also induce necroptosis after caspase blockage or starvation [125]. Even the induction of p53 transcription and the Bcl-2 family of proteins have been associated with necrosis, being BAX and BAK required for mitochondrial dysfunction in response to necroptotic agonists [128]. As another example of this complex interplay among cell death pathways, studies have shown that apoptosis and autophagy are activated in response to metabolic stress and that both autophagy and apoptosis are induced in response to ER stress, with the increase in autophagy being a contributing factor to ER-induced apoptosis [129].

In the IBD field, nevertheless, most studies evaluate these pathways in the context of bowel inflammation as isolated cell death mechanisms. In the case of autophagy, at least two different genes were found to be related to IBD through genome-wide association studies [16]. In mechanistic studies in vivo (both humans and mice) and in vitro, with extensive use of novel animal models, potential roles for apoptosis and necroptosis in the pathogenesis of these diseases have been also suggested. Recent studies point towards the existence of a complex crosstalk between autophagy/apoptosis, microbe sensing, and enhanced ER stress in the epithelium in the pathogenesis of CD [108]. Exciting new data indicate that the ileal involvement in CD might be related to a disturbance in Paneth cell function, establishing a link between innate immunity, ER stress, and cell death [108, 109]. In addition, necroptosis, a relatively novel programmed necrosis-like pathway associated with TNF receptor activation, also seems to play a role in the pathogenesis of CD and in specific experimental models of intestinal inflammation [47, 48]. Moreover, a stress-inflammation amplification loop mediated by DAMPs has been directly associated with cell death in the intestinal mucosa in both experimental models and human IBD [28, 29]. The cell death history in IBD seems to be an interesting example of data coming from huge hypothesis-free GWAS studies leading to hypothesis-driven mechanistic discoveries.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Tiago Nunes and Claudio Bernardazzi contributed equally to this work.

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Review Article

Cell Death and Deubiquitinases: Perspectives in Cancer

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The process of cell death has important physiological implications. At the organism level it is mostly involved in maintenance of tissue homeostasis. At the cellular level, the strategies of cell death may be categorized as either suicide or sabotage. The mere fact that many of these processes are programmed and that these are often deregulated in pathological conditions is seed to thought. The various players that are involved in these pathways are highly regulated. One of the modes of regulation is via post-translational modifications such as ubiquitination and deubiquitination. In this review, we have first dealt with the different modes and pathways involved in cell death and then we have focused on the regulation of several proteins in these signaling cascades by the different deubiquitinating enzymes, in the perspective of cancer. The study of deubiquitinases is currently in a rather nascent stage with limited knowledge both *in vitro* and *in vivo*, but the emerging roles of the deubiquitinases in various processes and their specificity have implicated them as potential targets from the therapeutic point of view. This review throws light on another aspect of cancer therapeutics by targeting the deubiquitinating enzymes.

1. Introduction

The balance between cell division and cell death is very important to coordinate normal cell turnover in both development and maintenance of tissue homeostasis in multicellular organisms [1]. The process of cell death has been selected evolutionarily over the years as an integral cellular mechanism [2] and any deregulation may lead to irregularities in embryogenesis, neurodegenerative disorders, and development of cancer [1]. Cell death may occur both normally or under certain pathological conditions. The existence of multiple death pathways is an in-built strategy to protect the organism against abnormalities arising in a single or multiple pathways, making the occurrence of diseases like cancer relatively rare, considering the large number of cell divisions and mutations incorporated during the lifetime of a multicellular organism [1].

With the advances in recent research over the last two decades new insights into the mechanisms and the factors involved in cell death have emerged, increasing its importance with respect to diseases. At the molecular level, cell death involves DNA damage, mitochondrial cytochrome c release, endoplasmic reticulum (ER) stress response, and so forth. The classification of the cellular death pathways is not discrete at certain instances and due to the presence of overlapping signaling pathways regulating these processes, there are a number of common factors involved, making the classification ambiguous. More than one death program may be activated at the same time and also there may be switching from one pathway to another depending on the context [3]. However, the basic mechanisms of cell death may be broadly classified as either suicide or sabotage [4]. In programmed cell death, the ultimate fate of the cell depends on the initiation signal and the degree of assault, and the outcome is known.

The programmed cell death processes involve well-coordinated factors and hence are subject to various modes of regulation. As in all cases the regulation may be at the gene, mRNA, or protein level. In this review, we focus on the post-translational modifications of proteins, specifically deubiquitination. We know that the process of ubiquitination is extremely dynamic, involving transient protein-protein interactions with high specificity and functions in

a highly context-dependent manner. Ubiquitination may be monomeric, polymeric, or multimonomeric, involving a variety of ubiquitin (Ub) linkages such as Lys 6 (K6), K11, K27, K29, K33, K48, K63, and Met1, each having specific functions [19]. This is a reversible process, involving deubiquitinating enzymes (DUBs) [20]. There are about 100 genes encoding DUBs in the human genome and this large number strongly suggests that distinct enzymes have highly specialized functions, but this study is still in a very nascent stage [21]. The basic features of DUB activity are processing of Ub precursors, editing of Ub chains, reversal of Ub conjugation, and recycling of Ub [22]. The specificity and activity of the DUBs is ensured by protein-protein interactions, the multiprotein complexes with which DUBs are associated, subcellular localization, phosphorylation, and changes in their expression or even differential activity in the various phases of cell cycle [21, 23]. Deubiquitinases regulate a variety of cellular processes by reversing ubiquitination.

DUBs are classified into six families: ubiquitin carboxy-terminal hydrolases (UCHs), ubiquitin specific proteases (USPs), ovarian-tumor proteases (OTUs), JAMM/MPN domain-associated metallopeptidases (JAMMs), Machado-Joseph disease protein domain proteases (MJDs), and monocyte chemotactic protein-induced proteases (MCPIPs). All these enzymes are cysteine proteases except the JAMMs. The largest family is the USPs with more than 50 members, all containing conserved domains and catalytic sites [20]. DUBs are often deregulated in cancers showing either mutations or altered expression levels [24]. Moreover, cancer cells are more sensitive to defects in protein folding and stability and E3 ligases are already being targeted for therapeutic purposes (e.g., bortezomib). But the DUBs are comparatively lesser in number and much more specific with respect to their functions and, hence, likely to be better targets [23, 24]. Recent research is bringing up first-generation inhibitors with specificity against either a single or a related group of DUBs [25]. Hence, the growing interest and importance of targeting DUBs bring the study of DUBs to high priority. In this review, we have tried to outline briefly the various programmed cell death pathways and their players and have also emphasized on the various deubiquitinases that are associated with these factors, focusing mainly on the context of cancer.

2. Importance of Cell Death in Cancer

2.1. Modes of Cell Death. The three main programmed cell death mechanisms are apoptosis, necrosis and autophagic cell death [26]. Several other modes of cell death have been reported such as paraptosis and mitotic catastrophe (see Figure 1). The different modes of cell death are not discrete at all times and there are frequent crosstalks. The outcomes may be a shared response of different modes of cell death but each one has its own salient features as described below. A comparative analysis of the different modes is illustrated in Table 1.

Apoptosis. Apoptosis may occur during embryonic development, in mature tissues like thymus or under pathological conditions. The cardinal features of apoptosis include membrane blebbing, rounding up of cells, reduction of cell volume, chromatin condensation, and nuclear fragmentation. This follows either a caspase-dependent or caspase-independent pathway, which may or may not be associated with mitochondrial and/or immunological involvement, based on intrinsic or extrinsic cues.

Necrosis. Necrosis generally occurs as a response to physical cellular injury and is mostly associated with pathological conditions. Necrosis is characterized by gain in cell volume, swelling of organelles, rupture of plasma membrane, and elicitation of inflammatory tissue response. Necrosis was initially thought to be an uncontrolled (accidental) death process, but recent evidences of well-defined signaling pathways involved in necrosis are coming into focus. Thus, programmed necrosis also known as "necroptosis," exists as a back-up system for the cell when apoptosis is inhibited [27].

Autophagic Cell Death. Autophagy is a prosurvival strategy for the cells in cases of stress like nutrient or growth factor deprivation or cytokine-induction. The mode of action is via sequestration of cytoplasmic material within autophagosomes for lysosomal degradation, with the absence of chromatin condensation, generally mediated by the autophagy genes (ATGs) [4, 28]. Hence, activation of autophagy under cellular stress has a cytoprotective outcome to maintain cellular homeostasis and inhibiting it may lead to cell death. This may again cause inhibition of developmental cell death indicating a role of autophagy in cell death. Therefore, the decision of whether autophagy results in cell survival or death depends on the context [29].

Pyroptosis. Pyroptosis involves caspase-1 mediated cell death, an atypical caspase-dependent mechanism seen in monocytes, macrophages, and dendritic cells in case of microbial infection with implications in host defence [26, 30].

Paraptosis. Paraptosis is cytoplasmic vacuolization initiated by swelling of mitochondria and ER. The response is mediated by mitogen-activated protein kinases (MAPKs) [31].

Mitotic Catastrophe. Mitotic catastrophe is a process occurring in the absence of complete mitosis. It is characterized by multinucleated enlarged cells [28] and generally marked as a cellular strategy to combat genomic instability, which is very common in cancer. The major factors involved are cell cycle-dependent kinases such as cyclin-dependent kinase 1 (cdk1), aurora kinase B, polo-like kinases (Plks); cell cycle checkpoint proteins (Chk1 and 2, p53, and Rb); Bcl-2 family proteins; and caspases [32].

Senescence. The outcome of senescence in cells can be visualized by tumor suppression or promotion, aging, and tissue repair, because the process is associated with inhibition of cell proliferation, aging, and cell death [33]. Cellular senescence can occur during irreversible cell cycle arrest upon encountering oncogenic stress, wherein cells become flattened, highly vacuolated, and heterochromatinized and form autophagosomes. The key players are PTEN, p53, p21, p16, and so forth [33]. In the somatic cells, telomere shortening

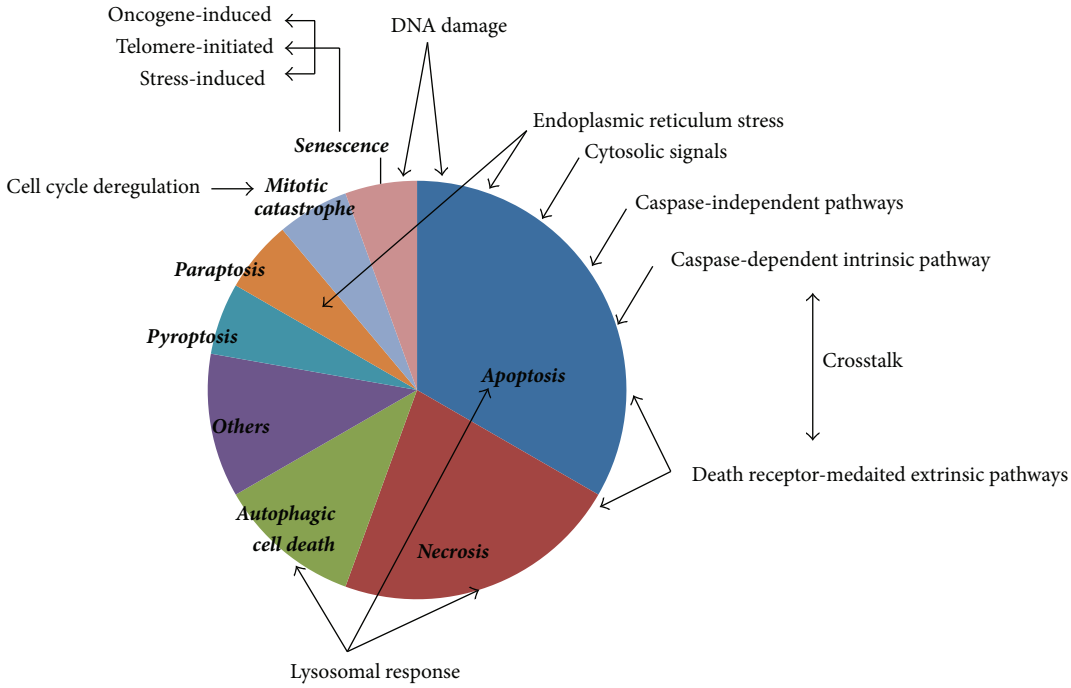

FIGURE 1: Relative occurrence of the various modes of programmed cell death. The figure illustrates the relative occurrence of different modes of programmed cell death (in bold and italics). The signaling mechanisms that are triggered as a cellular response leading to a specific mode of cell death are also represented in the figure (normal text and arrows pointing towards the process).

occurs with each replicative cycle, leading to replicative senescence and ultimately cell death which may be partially due to elicitation of DNA damage response signaling. It is the normal process of aging resulting from loss of clonogenic potential. But almost 85% of human cancers show enhanced expression of telomerases [34] accounting partially for immortalization of the cancer cells. Culture stress, like substrata, serum, oxidative stress, and so forth, may also lead to senescence in *in vitro* settings [33, 35].

2.2. Pathways Involved in Cell Death and Their Components. For better understanding of the molecular mechanisms of the various modes of cell death mentioned above, here we have discussed the different pathways and the factors that are the main players involved in executing the cellular fates (also see Figure 1). As mentioned earlier, several signaling pathways are common in case of the cell death pathways and these involve various common players. The cellular response elicited may also overlap in certain cases. Hence, in this section, we have described the pathways one by one and intermittently discussed the involvement of the organelles in the specific contexts.

2.2.1. Intrinsic Cell Death Pathways. The intrinsic death pathways are triggered by internal cellular cues and can generally be classified on the basis of their caspase dependency. Varied mitochondrial events remain associated with the ultimate outcome in either case.

Caspase-Dependent Intrinsic Apoptotic Pathway. The intrinsic pathway is initiated by intrinsic stimuli, like DNA damage, overload in cytosolic calcium, cellular starvation, oxidative or radiation or cytotoxic stress, and so forth, resulting in mitochondrial events determined by the Bcl-2 family proteins which have opposing roles: proapoptotic, Bax, Bak, Bad, Bcl-X_S, Bid, Bik, Bim, and Hrk (cause mitochondrial damage) while antiapoptotic, Bcl-2, Bcl-X_L, Bcl-W, Bfl-1 and Mcl-1 antagonize them [36, 37]. In humans, twelve caspases have been identified [38]. These are present as inactive zymogens, cleaved to produce the active caspases upon specific stimuli and function in a hierarchical fashion starting from the upstream initiator caspases (2, 8, 9, and 10) to the downstream executioner caspases (3, 6, and 7). Caspase 9 initiates mitochondrial pathways while caspase 8 and 10 trigger the death receptor mediated pathways. Under early apoptotic conditions, DNA fragmentation initiates caspase-mediated poly-ADP-ribose-polymerase (PARP) cleavage, binding the DNA fragments and blocking the access of DNA repair enzymes leading to apoptosis [39].

Caspase-Independent Intrinsic Cell Death Pathways. Calcium-activated calpain promotes release of apoptosis inducing factor (AIF) from mitochondria [40] and AIF translocation to the nucleus which requires PARP-1 activity [41], leading to apoptosis in a caspase-independent manner. Endonuclease G (endo G) may also participate in caspase-independent cell death pathways. Endo G is released from mitochondria under apoptotic stimuli like UV radiation or use of anti-Fas antibodies, to translocate to the nucleus, wherein it cleaves chromatin DNA into nucleosomal fragments. Endo G acts in cooperation with exonucleases and DNase I, to facilitate DNA

Table 1: Comparative analysis of the different modes of cell death.

Feature	Mode						
	Apoptosis	Necrosis	Autophagic cell death	Pyroptosis	Paraptosis	Mitotic catastrophe	Senescence
Programmed	Yes	Yes	Yes	No	Yes	No	No
Change in cell volume	Reduction	Increases	—	—	—	Increases	—
Chromatin condensation	Yes and nuclear fragmentation	No	No	—	—	Micronucleation	Heterochromatinization
Organelle status	Generally unaltered	Swelling of organelles	Lysosomal rupture	—	Mitochondria and ER swelling	Multinucleated cells	—
Change in membrane dynamics	Blebbing and PS flipping	Ruptured	—	—	—	—	—
Cytoplasmic material	No sequestration/release	Released due to cell rupture	Sequestered by autophagic vacuolization	—	Vacuolization seen	—	Vacuolization seen
Mitochondrial response	Occasionally	Occasionally	Occasionally	—	—	Occasionally	No
Caspase dependence	In some cases	In some cases	In some cases (caspase-1)	In some cases (caspase-1 or 7)	No	In some cases (caspase-2)	—
Immunological response	Rarely	Yes	No	—	—	—	—
Inflammatory response	Generally no	Yes	—	Yes	—	—	—
ER stress response	Yes	—	Yes	—	—	—	—
DNA damage response	Yes	Yes	Yes	—	—	Yes	Yes
Occurrence	During development, in adult tissues and pathological conditions	Physical injuries and pathological conditions	Stress response and development	In microbial infection as host defence	At times overlaps with other PCDs	Triggered by mitotic failure	Mainly stress-induced, during aging, and may involve telomere-shortening

processing. This nuclease activity may be found even in the presence of caspase inhibitors.

Granzyme A (Gzm A) also plays a role in caspase-independent apoptosis caused by massive single-stranded DNA nicking. Gzm A induces loss of mitochondrial inner membrane potential and generates reactive oxygen species (ROS). In the nucleus, ROS cleaves three members of the ER-associated DNA repair SET complex (HMG2, APE1, and SET). As a consequence, DNase NM23-H1 is activated, along with enhanced activity of other DNases due to destabilization of nuclear lamins and histone H1 [42]. Gzm C/H and K function similar to Gzm A, but their roles have not yet been fully identified [43].

Mitochondrial Response. The mitochondrial involvement in cell death may occur under cellular stress like nutrient deprivation or hypoxia, DNA damage, or activation of oncogenes leading to deregulated cell cycle and evasion of cell cycle checkpoints triggering aberrant cell death pathways. The Bcl-2 family proteins (Bax and Bak) oligomerize, leading to mitochondrial outer membrane permeability (MOMP). This is considered as the state of "no return." This leads to the release of cytochrome C and/or AIF and endo G to the cytosol. Cytochrome C interacts with the apoptotic protease activating factor 1 (Apaf-1) to form the apoptosome complex [44] and subsequently activating the caspase pathway (procaspase 9, followed by caspases 3, 6, and 7). AIF and endo G trigger caspase-independent pathways. Sometimes free radicals are generated due to uncoupling of oxidative phosphorylation and diversion of electrons from the respiratory electron transport chain [45, 46]. Other mitochondrial proteins like inhibitors of apoptosis (IAPs) can inhibit the caspases via direct interaction; for example, XIAP (X-linked IAP) inhibits caspases 9, 3, and 7 [47] or IAP antagonists like SMAC/DIABLO (second mitochondrial activator of caspases/direct IAP binding protein with Low pI) or Omi/HtraA2 bind IAPs, preventing their function and favoring caspase activation.

Endoplasmic Reticulum (ER) Stress-Induced Intrinsic Pathway. ER stress induced by altered calcium homeostasis, glucose starvation, hypoxic stress, low redox potential, excessive or defective protein synthesis/secretion, and so forth may lead to apoptosis [48]. Upon accumulation of unfolded proteins in the ER lumen, unfolded protein response (UPR) is set off, consisting of reduction in global protein synthesis, induction of chaperones and proteins related to protein folding, and translocation of improperly folded proteins from the ER to the cytosol for proteasomal degradation [49]. Prolonged ER stress may induce autophagy (discussed later in Section 3.5); activation of "caspase 8-Bid-cytochrome C release" axis via the mitochondrial pathway (described later in the "crosstalks" subsection) [50]; or calpain-mediated activation of caspase 12 further activating caspase 9 [51].

2.2.2. Extrinsic Cell Death Pathways. The extrinsic pathways are generally initiated by external stimulation of the death family of receptors and, hence, this mode is also known as the death receptor mediated extrinsic death pathway. The death receptors (DR) are a family of six members containing a conserved death domain (DD): Fas/CD95/APO-1, tumor necrosis factor receptor 1 (TNFR1), DR 3, TNF apoptosis-inducing ligand (TRAIL) R1/DR4, TRAIL R2/DR5, and DR6. Signal transduction via these receptors depends on the cellular context and stimulus, determining the outcome, which may be prosurvival, proinflammatory, apoptotic, necrotic, and so forth. The two death domain associated adaptor proteins involved here are FADD (Fas associated death domain) and TRADD (TNF receptor associated death domain). Signaling through FADD results in apoptosis while involvement of TRADD may have both apoptotic as well as nonapoptotic outcomes [52].

Extrinsic Apoptotic Cascade. Binding of TNF family ligands (FasL and TRAIL) to the death receptors (Fas, TNFR1, etc.) at the plasma membrane leads to recruitment of FADD, receptor interacting protein kinase 1 (RIP1) and procaspase 8 to form the death-inducing signaling complex (DISC). DISC formation triggers caspase 8 activation, ubiquitination, and degradation of RIP1 followed by caspase 3 activation and induction of apoptosis [53]. Fas may also associate with another DD associated protein Daxx (DD associated protein 6) inhibiting the FADD induced pathway and triggering JNK signaling. This leads to the induction of another discrete apoptotic cascade [54].

Extrinsic Nonapoptotic Cascade. When TNF-α binds to TNFR1, TRADD is recruited, leading to the formation of two distinct complexes, I and II [55, 56]. Complex I contains TRAF2 and 5 (TNF receptor-associated factors 2 and 5), RIP1, cIAP1 and 2; polyubiquitinating RIP1 by linking K63-Ub chains; recruiting TGF-β activated kinase 1 (TAK1), TAK1 binding protein 2 (TAB2), nuclear factor kappa B (NFκB) essential modifier (NEMO) and I-kappa B kinase (IKK); activating NFκB and leading to expression of anti-apoptotic proteins, such as IAPs, and cFLIP (cellular FLICE-like inhibitory protein), and cell survival [57]. Later on, TNFR1 is internalized, leading to formation of a cytosolic complex (complex II) containing TRADD, FADD, RIP1 and 3, and procaspase 8, initiating either the extrinsic apoptotic cascade or truncating Bid (see the details later) to trigger the mitochondrial pathway. Hence, the balance between these two complexes leads to the differential outcomes [58].

Extrinsic Necrotic Cascade or Necroptosis. When the caspases are inactivated, a pronecrotic ripoptosome complex [59] similar to DISC is formed containing an additional member RIP3. RIP1 and 3 are activated and RIP1-RIP3 complex formation triggers production of mitochondrial ROS, PARP-1 cleavage, activation of calpains, and so forth, leading to programmed necrosis [60]. Again, DNA damage activates PARP-1, which elicits TRAF2 and RIP1 mediated JNK-1 activation to induce mitochondrial AIF release and translocation to nucleus, leading to necrotic cell death [61].

2.2.3. Autophagic Cell Death and Lysosomal Response. Bcl-2 family of proteins has also been implicated in autophagic cell death, wherein, Bcl-2 can bind to Beclin-1 (a haploinsufficient

tumor suppressor) and inhibit its activity. This suppresses autophagy and leads to tumorigenesis [62]. UVRAG (UV radiation resistance associated) and Bif-1 (Bax-interacting factor 1) are positive regulators of Beclin-1 promoting autophagy [63]. Bif-1 may also act via its interaction with proapoptotic Bax [64]. During starvation, Bcl-2 is phosphorylated and Beclin-1 is released inducing autophagic cell death. PARP-1 cleavage may also be associated with autophagic cell death [65]. Lysosomes are essential for autophagy. The rupture of lysosomes releases cathepsins (acid hydrolases) to the cytosol pushing cells to apoptosis or necrosis. The cathepsins trigger both caspase-dependent mitochondrial response as well as caspase-independent activation of Bax and release of AIF [66]. Some cathepsins also induce Bid mediated apoptosis [67].

2.2.4. Crosstalks. In some cases, extrinsic signals may lead to low DISC formation, leading to Bid cleavage by activated caspase 8, changing the MOMP. This results in mitochondrial translocation of truncated Bid (tBid), cytochrome C release, apoptosome formation, and triggering of downstream caspase cascade, linking the intrinsic and extrinsic apoptotic pathways [50]. Formation of tBid in the cell may also result from Gzm B mediated Bid cleavage ultimately resulting in apoptosome formation as described above. Unlike the other granzymes mentioned earlier, Gzm B can also trigger a caspase-dependent apoptotic cascade and is known to cleave and activate caspases 3 and 8, while cleavage of prosurvival protein Mcl-1 leads to its inactivation [43].

DNA damaging agents may induce cell cycle arrest and induce autophagy and mitophagy, by delaying apoptosis. Both apoptosis and autophagy are regulated by the Bcl-2 family proteins [68]. Hence, apoptosis and autophagy may show synergistic effects at times, while at other times, suppression of apoptosis may lead to autophagy. Although not very clear, the two processes may be regulated by discrete DNA damage response pathways and the Bcl-2 family proteins play a crucial role in maintaining this balance. Inactivation of caspases may lead to necroptosis instead of apoptosis, while presence of necrostatin-1 (a specific inhibitor of necroptosis) may lead to reversal of necrosis to apoptosis. Sometimes programmed necrosis and autophagic cell death may occur simultaneously [60].

2.2.5. Compartment-Specific Responses in Cell Death Pathways

Nuclear Response. The response elicited upon nuclear DNA damage is mainly via p53 activation, either triggering the transactivation of a number of Bcl-2 family proteins (Bad, Bid, Puma, and Noxa) which are effectors of mitochondrial destabilization or activation of caspase 2 and further inhibition of NFκB signaling, both leading to apoptosis. Upon genotoxic stress, most commonly double-strand breaks (DSBs) activate the ATM (ataxia-telangiectasia mutated) and ATR (ataxia-telangiectasia mutated and Rad3-related) kinases, which phosphorylate and stabilize p53, blocking its ubiquitination by Mdm2 [69]. The outcome may be either G1 or G2 cell cycle arrest due to stabilization of p21 or apoptosis due to upregulation of Bax or PUMA [70]. Other kinases, like Plk-3, homeodomain interacting protein kinase 2 (HIPK-2), may also phosphorylate p53 giving apoptotic outcomes [71].

Cell cycle is a highly regulated process with multiple checkpoints arresting cells at G1/S, intra S, G2/M, mitotic spindle assembly; either for DNA repair or sending cells to death pathways if the damage is extreme; or even forcing cells to enter quiescence by exiting cell cycle (G0 phase) during starvation. Cell cycle arrest may become irreversible sending cells to senescence [72]. Rb controls the G1 checkpoint and is phosphorylated by an array of cdks to be inactivated, for transition from G1 to S phase. The mitotic checkpoint is maintained by Mad, Bub, aurora kinases, and Plks, to check the mitotic spindle formation. To circumvent aberrations in this phase, cells undergo mitotic catastrophe. Loss of control of the checkpoints leads to genomic instability, providing adaptive or selective advantage to the cancer cells [73].

Cytosolic Response. Akt is an important oncogenic kinase which is one of the master regulators acting upstream of many pathways involved in cell survival, proliferation, death, transcription, translation, and so forth [74]. Phosphorylation of forkhead box proteins (FOXOs) by Akt leads to their nuclear exclusion repressing proapoptotic genes—p27, Bim, and FasL. Other direct proapoptotic targets of Akt are Bad, caspase 9, Mdm2, and GSK-3β [75]. IKK activation induces NFκB signaling to transcribe antiapoptotic proteins—Bcl-X_L, XIAP, and so forth [76]. All the above processes inhibit apoptosis. One of the negative regulators of Akt is phosphatase and tensin homolog (PTEN), which is often deleted or mutated in cancers. While under nutrient deprivation, mTOR complex 1 is inactivated and autophagic response is initiated. Therefore, deregulation of Akt pathway is another strategy of the cancer cells to gain chemoresistance.

2.3. Perspectives in Cancer. Evasion of apoptosis is one of the hallmarks of cancer [77] and alterations in the apoptotic cascade may result in changes in tumor development and cancer progression [78]. Malignant cells inactivate the endogenous inducers of cell death as a strategy to block the natural cell death pathways, providing a selective survival advantage to the cancer cells [79]. There are several examples where targeting the cell death pathways in regulating the process of oncogenic progression has been used as a strategy for drug development by restoration of the endogenous autodestruction pathways [79–81]. Most of the chemotherapeutic drugs act by triggering the cell death pathways in the tumor cells [82]. A major part is by inducing apoptosis, either via the mitochondrial pathways [83] or by stimulation of the death receptor pathways [84], although involvement of the other modes of cell death has also been reported [85, 86]. Apoptosis and autophagic cell death do not elicit any immune response in the cells and hence are preferred over necrosis. While both of these processes are often found to be defective in cancer, necrosis may be found in tumors. A possible explanation may be elicitation of a persistent cytokine production helping in tumor growth leading to poor prognosis [87]. Cancer cells adapt to hypoxic stress and activate stress response

pathways involving the hypoxia-inducing factors (HIFs), inducing autophagy at the hypoxic core and promoting cell survival. The response mostly depends on the dose of the chemotherapeutic stress and on the cell type [1]. Some of the strategies have been discussed below.

2.3.1. Targeting Bcl-2 Family Proteins. Natural compounds, synthetic antagonists, and analogs of Bcl-2 family members have been used to regulate cell death pathways. Bid and Bax are subject to ubiquitin mediated degradation attenuating apoptosis in cancer cells (example, mitochondrial Bax is degraded in PCa cells). While this strategy is utilized by cancer cells, it has also been exploited for therapeutic purposes; for example, inhibition of the proteasomal system sensitizes chronic lymphocytic leukemia (CLL) cells to TRAIL-induced apoptosis [88].

2.3.2. Targeting the Caspases. The cancer cells follow three mechanisms to negate the effect of caspases: preventing the activation of the procaspases, neutralizing active caspases, and regulating the gene expression of either caspases or their activators. There are eight members in the IAP family in humans which can directly bind to the caspases and either block their activity or mark them for ubiquitin mediated degradation. These IAPs are frequently found to be upregulated in cancers [89]. For example, c-FLIP suppresses TNF-α induced apoptosis via caspases 8 and 10; CARD8 (caspase recruitment domain-containing protein 8) binds to procaspase 9; XIAP inhibits caspases 3, 7, and 9. Natural antagonists of the caspases such as SMAC/DIABLO and Omi/HtrA2 compete with caspases to bind IAPs [90].

2.3.3. Targeting the Tumor Suppressors. In cancers, p53 pathway is frequently inactivated by either p53 mutations or Mdm2 overexpression. In such cases, DNA damage response is elicited via ATM and ATR kinases which regulate the Chks, in turn, activating NFκB, Akt, survivin, and so forth [91]. Cancer cells have evolved strategies to counteract these basic cellular mechanisms to deregulate the cell cycle and facilitate either cancer cell growth or to evade cell death. Frequent inactivating mutations or deletions in tumor suppressors like PTEN, p53, Rb, BRCA-1 and 2, p16, and ATM are associated with cancers. Premature senescence has been reported as a drug-induced tumor suppressive mechanism having a potential in cancer treatment [92]. Some tumor suppressors induce autophagy, for example, Beclin 1, UVRAG, PTEN and Bcl-2, while some oncogenic proteins like mTOR inhibit autophagy. p53 displays a dual role by both inducing and inhibiting autophagy.

3. DUBs Involved in Cell Death Associated Pathways Related to Cancer

As DUBs are integral regulatory molecules of most of the cellular functions, it has high implications in proper functioning of the cellular machineries (elaborated in other reviews [20, 21, 23, 24, 93–95]). Deubiquitinating enzymes negatively regulate the ubiquitin signaling pathway and influence both oncogenes and tumor suppressors. Due to their varied substrates, the nature of the DUBs always remains dual (both oncogenic and tumor suppressive) and their function is largely tissue-specific and context-dependent. Some of the cellular processes that are integrally related to cell death include cell cycle, DNA damage response and repair, and other signaling pathways. In this review, we emphasize on the different DUBs involved in these pathways (briefly outlined in Table 2 and Figure 2).

3.1. Cell Cycle. The process of cell division goes on simultaneously with cell death processes, establishing several links. Any deregulation in the continuous cycling may lead to cell cycle arrest, senescence, or death. Many DUBs such as USP7, USP13, USP39, USP44, CYLD (cylindromatosis), and BAP1 (BRCA1 associated protein-1) are associated with the different phases of cell division.

3.1.1. G1, S, and G2 Phases. USP2 stabilizes cyclin D1 by direct interaction [96]. USP7 or HAUSP (herpesvirus-associated ubiquitin-specific protease) deubiquitinates SCF-β-TrCP mediated K48-linked Ub chains on claspin, the upstream regulator of Chk1 [97]. USP13 counteracts S phase kinase-associated protein 2 (Skp2) ubiquitination via the anaphase promoting complex/cyclosome (APC/C^{Cdh1}), delaying cell cycle by accumulation of p27 [98]. USP19 deubiquitinates Kip1 ubiquitination-promoting complex protein 1 (KPC1) regulating $p27^{Kip1}$ [99] and some KPC1 independent cell cycle regulation also exists [100]. UCH-L1 colocalizes with Jab1 sending $p27^{Kip1}$ to proteasomal degradation, prevents senescence, and ensures proper somatic cell division [101]. USP17L2 deubiquitinates cdc25a, promoting oncogenic transformation [102].

3.1.2. Spindle Assembly and Mitosis. USP39 deubiquitinates aurora B kinase maintaining spindle assembly checkpoint integrity [103] while USP44 stabilizes Mad2/Cdc20 complex inhibiting premature activation of the APC/C^{Cdh1} complex [104].

3.1.3. G1/S and G2/M Checkpoints. USP7 regulates multiple cell cycle checkpoints, via deubiquitination of p53 [105] and Rb [106] or their negative regulator Mdm2 [107]. USP4, reported as an oncogenic protein, is known to interact with the pocket proteins (Rb, p107, and p130) although no deubiquitinating activity has been reported [108]. USP7 deubiquitinates checkpoint with forkhead and RING finger domains protein (Chfr), which in turn ubiquitinates histone deacetylase 1 (HDAC1), leading to upregulation of $p21^{Cip1/Waf1}$ and induction of G1 arrest [109]. Cdk2 activates USP37 which also antagonizes APC/C^{Cdh1} complex, deubiquitinating cyclin A and entry into S phase, another G1/S checkpoint [110]. BAP1 also controls G1/S cell cycle progression by regulating BRCA-1 [111], Ying Yang 1 (YY-1), and host cell factor 1 (HCF-1) [112]. CYLD regulates Plk-1 [113] protecting G2/M checkpoint.

Table 2: DUBs and their substrates involved in cell death pathways.

Deubiquitinase	Substrate	Hydrolyzes Ub-linkage	Relevance in cell death
Ubiquitin specific proteases			
USP1	FANCD2	—	Activates Chk1
	PCNA	—	
USP2	PHLPP	K48	Inhibits Akt to induce apoptosis
	Cyclin D1	K48	Cell cycle progression
	Mdm2	K48	Inhibition of p53
	MdmX	K48	Inhibition of p53
USP2a	Fas	K63	Inhibition of NFκB signaling
	RIP1	K63	Inhibition of NFκB signaling
	TRAF2	K63	Inhibition of NFκB signaling
USP3 + BRCC36	Rap80	K63	Maintain G2/M checkpoints on DSBs
USP4	Rb, p107, p130	Associates	Cell cycle arrest
	TCF4	—	Suppresses β-catenin transcription
USP7 (HAUSP)	p53	K48	p53 stabilization
	PTEN	K63	PCa progression
	p53	K48	Apoptosis
	Rb	K48	Differential regulation
	Mdm2/MdmX	K48	Inhibits p53
	Daxx	—	Regulates Mdm2 activity under stress
	p53/Mule	—	Indirect regulation of p53
	H2B, Mdm2	—	Regulates chromatin remodelling
	Tip60	K48	p53 dependent apoptosis
	Chfr	—	Enhanced ubiquitination of HDAC1 and upregulation of p21
	ERCC6	—	Stabilizes RNA Pol II-ERCC6 complex
	Claspin	K48	Chk1 regulation
	FOXO 3a and 4	K63	Accumulation of p27
	RIP1	K63	Positive regulation of TNF-α induced apoptosis
	p65-NFκB	—	Upregulates NFκB target gene transcription
USP8	ErbB3 via Nrdp1	—	Activation of EGFR pathway
USP9X	Mcl-1	K48	Radioresistance
USP10	p53	K48	Stabilizes p53
USP11	IκB	—	Inhibition of NFκB signaling by sequestering NFκB in the cytoplasm
USP12	PHLPP	K48	Inhibition of Akt to induce apoptosis
USP15	APC	K48	Promotes β-catenin
	IKK-α	K48	Inhibition of NFκB signaling and activation of p53
USP19	KPC1	—	Accumulation of p27
USP21	RIP1	K63	Inhibition of NFκB signaling
USP28	cMyc	K48	Reverses FBW7-α mediated ubiquitination
	Chk2	K48	Chk2-p53-PUMA apoptosis
USP29	p53	K48	Stabilization of p53
USP37	Cyclin A	K48	Induction of G1/S
USP39	Aurora B	—	SAC integrity
USP42	p53	K48	Enhances p53 stability
USP44	Mad2/cdc20	—	Inhibits APC/C^{cdh1} complex
USP46	PHLPP	K48	Inhibition of Akt to induce apoptosis
USP47	DNA Pol β	—	BER response
USP50	Wee1	K48	Prevents mitotic entry

Table 2: Continued.

Deubiquitinase	Substrate	Hydrolyzes Ub-linkage	Relevance in cell death
		Ubiquitin carboxy-terminal hydrolases	
UCH-L1	Jab1	Colocalizes	Inbihits p27
BAP1	BRCA-1	—	Induction of G1/S
	YY-1	—	Induction of G1/S
	HCF-1	—	Induction of G1/S
	Plk-1	—	G2/M protection
CYLD	NEMO	K63	Inhibition of NFκB signaling
	TRAF 2, 6 and 7	K63	Inhibition of NFκB signaling
	RIP1	K63	Inhibition of NFκB signaling
	TAK1	K63	Inhibition of NFκB signaling
	BCL-3	—	Induction of p50-BCL3 and p52-BCL3 complexes inhibiting cell proliferation
		Ovarian tumor proteases	
A20	RIP1	K63	Inhibition of NFκB signaling
	TRAF6	K63	Inhibition of NFκB signaling
	Caspase 8	K63	Regulates caspase 8 activity
OTUB1	Ubc13	Associates	Inhibits RNF168
OTUD5	p53	K48	Induction of apoptosis
Cezanne (OTUD7B)	RIP1	K11	Inhibition of NFκB signaling
	EGFR	—	Enhances EGFR signaling
TRABID/ZRANB1	APC	K63	Induction of TCF4/β-catenin transcription
		JAMM/MPN domain-associated metallopeptidase	
AMSH	EGFR	K63	Regulates endocytic trafficking of EGFR
BRCC36	H2A, H2AX	K63	At sites of DSBs
POH1	ErbB2	—	Regulates EGFR signaling
		Monocyte chemotactic protein-induced proteases	
MCPIP1	RIP1	K63	Inhibition of NFκB signaling
	TRAF2 and 6	K63	Inhibition of NFκB signaling

USP50 regulates HSP90-dependent Wee1 stability preventing mitotic entry, acting as another G2/M checkpoint [114].

3.2. DNA Damage and Repair. DNA damage and high mutation rates are responsible for genomic instability in cancer cells. DNA damage triggers DNA repair pathways and some of the major ones in the mammalian system are mismatch repair (MMR), double strand break (DSB) repair, base excision repair (BER), nucleotide excision repair (NER), homologous recombination (HR) repair, nonhomologous end joining (NHEJ), translesion DNA synthesis (TLS), and so forth. The different pathways exist to combat the insults from a variety of DNA damage stimuli and this is known as cellular DNA damage response. These pathways are highly subject to regulation by the UPS and DUBs [115]. When the cells reach a state of chronic damage, that is, the point of no return, several other cellular responses are elicited such as apoptosis, autophagic cell death, and senescence [68].

3.2.1. Double Strand Break Repair. DSB repair pathway is initiated by recruitment of BRCA-1 and p53 binding protein 1 (TP53BP1). K63-linked ubiquitin accumulates on Rap80 at the DSB foci with the concerted effect of RNF8, RNF168, and Ubc13, which are clipped off with the assistance of USP3 and BRCC36 to maintain the G2/M checkpoint. BRCC36 also hydrolyzes K63-linked Ub chains form H2A and H2AX at the sites of double strand DNA damage [116, 117]. Although OTUB1 is not catalytically involved in deubiquitinating these K63-linked chains, it may interact with Ubc13 and inhibit the E3 ligase RNF168 [118]. USP11 plays a role in the HR in response to DNA damage induced by DSBs caused by agents like bleomycin, mitomycin C, cisplatin, and so forth [119]. Although there is no evidence of BRCA-2 deubiquitination by USP11, the interaction may be involved in recruiting USP11 to the damage site [120]. USP28 stabilizes Chk2 in Chk2-p53-PUMA pathway inducing apoptosis [121]. OTUD5 also helps in stabilizing p53 and inducing apoptosis upon DNA damage signals [122].

3.2.2. Base Excision Repair. BER mechanisms are elicited by genomic instability arising from DNA base lesions. DNA Pol β is a component of the BER complex. It is ubiquitinated by Mule and CHIP and is deubiquitinated by USP47 [123]. USP7 plays multiple roles in BER. It promotes BER by regulating chromatin remodelling by deubiquitination of H2B, though this activity was shown *in vitro*, this activity was also seen as

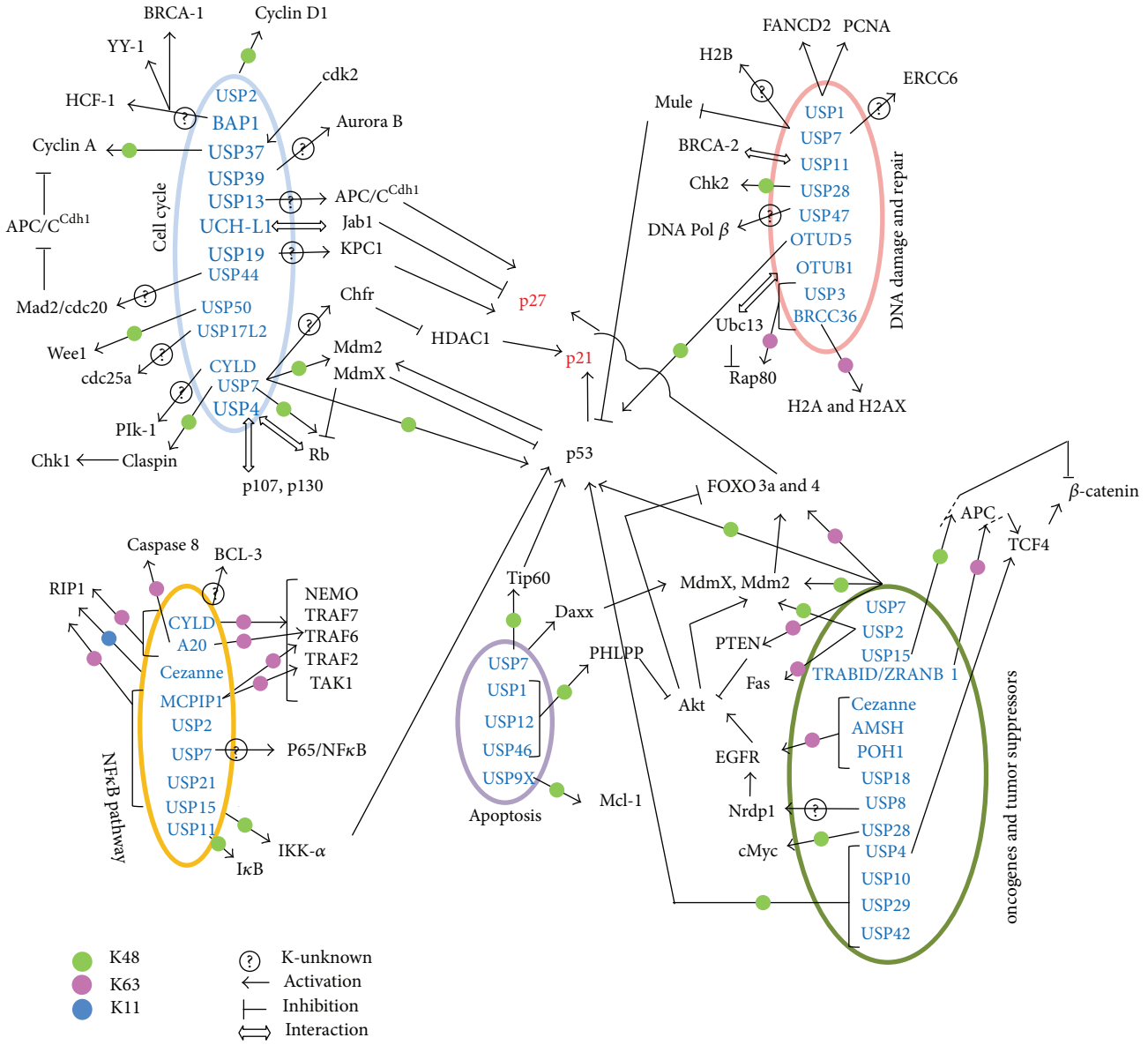

Figure 2: DUBs regulating cell death pathways. The figure illustrates the deubiquitinases regulating different pathways involved in cell death and cell growth in the perspective of cancer cells. The DUBs remove K48 (green), K63 (pink), and K11 (blue) linked Ub chains from their substrates. In some cases, the specific linkage remains unknown (?). The role of some of the DUBs is unclear in terms of deubiquitination of the substrate, but there is interaction (indicated by ⇔). Activation is represented by (→) and inhibition by (⊤).

an indirect result of Mdm2 deubiquitination by USP7 [124]. Also, DNA damage induced dephosphorylation of USP7 subjects Mule (an E3 ligase for p53) to self-ubiquitination and degradation, stabilizing p53 and activating the damage repair pathway [125]. In this context, it might be worthwhile to mention that post-translational modifications are very important in BER pathways. Apart from phosphorylation and acetylation, AP endonuclease (APE1) is also ubiquitinated, with the help of Mdm2 for degradation. This is a point of crosstalk between p53 and BER pathways [126]. As USP7 is a crucial factor in regulating the p53-Mdm2 balance in the cells, it may be speculated to play yet another role in BER response via modulation of APE1.

3.2.3. Nucleotide Excision Repair. During UV radiation-mediated damage, stalling of RNA Pol IIo at DNA lesion sites is a signal for apoptosis and its removal or degradation allows the access to NER machinery. The RNA Pol II cofactors are UV-sensitivity scaffold protein A (UVSSA), ERCC6, and ERCC8. USP7 is an additional cofactor in the complex and stabilizes ERCC6 [127].

3.2.4. Crosslink Repair. This mechanism involves PCNA (proliferating cell nuclear antigen) and FANCD2 (Fanconi anemia, complementation group D2) and acts at the site of fork-blocking lesions arising from interstrand crosslinks. USP1 deubiquitinates both PCNA and FANCD2 [128, 129].

Response to cell damage is generally under the control of ATM/ATR and Chk1 and 2. FANCD2 stabilization leads to activation of Chk1, the initial step in DNA damage repair [130, 131]. A number of DUBs, such as USP15, USP19, USP28, and USP34 [132, 133], act at the interface of DNA damage and cell cycle progression by DNA repair to decide the cell fate [25].

3.3. Apoptosis. Both apoptosis promoting and suppressing roles are displayed by the various DUBs linked to the apoptotic pathways. USP2, USP7, USP8, USP9X, USP15, USP16, USP17, USP28, CYLD, UCH-L1, A20, and so forth promote apoptosis while USP2, USP7, USP9X, USP18, and so forth suppress apoptosis [20]. USP7 deubiquitinates and stabilizes the acetyltransferase Tip60 to induce p53-dependent apoptotic pathways [134]. The opposing roles played by USP7 and Mdm2 is critical for maintaining the level of Daxx in the cancer cells [135]. In colon adenocarcinoma cells, the WDR48 and USP12 complex deubiquitinates PHLPP1 (PH domain and leucine rich repeat protein phosphatase 1) to enhance its stability, hence negatively regulating Akt activation and promoting cellular apoptosis [136]. USP1 [137] and USP46 [138] are other DUBs known to deubiquitinate PHLPP1, with a similar outcome in other cancers as well. Radiation-induced activation of USP9X deubiquitinates Mcl-1, inhibiting its degradation and apoptosis, conferring radioresistance [139].

3.4. Signaling Pathways Involving Key Oncogenes and Tumor Suppressors. The decision of cell death or aberrant growth leading to tumorigenesis is an outcome of the imbalance in the regulation of oncogenes and tumor suppressors. So here we have indicated the different DUBs regulating these processes.

3.4.1. Signaling through Receptor Tyrosine Kinases (RTKs). RTKs are important upstream factors in oncogenic signaling cascades and also targets for drug development. These are frequently internalized, ubiquitinated, and sorted in the endosomes leading either to lysosomal degradation or change in subcellular localization like nuclear translocation. These processes are prone to multiple aberrations in cancers with varying outcomes. AMSH (associated molecule with the SH3 domain of STAM) expression is elevated in many cancers and is capable of hydrolyzing K63-linked Ub chains from epidermal growth factor receptor (EGFR) recycling it to the plasma membrane [21, 95]. Cezanne-1 deubiquitinates and stabilizes EGFR, enhancing EGFR signaling and cancer progression [140]. USP18 also regulates EGFR [141]. POH1 regulates ErbB2, an EGFR family member [95]. USP8 regulates another EGFR family member, ErbB3 by modulating Nrdp1 (neuregulin-receptor-degradation protein-1) [142].

3.4.2. Wnt/β-Catenin Signaling. DUBs regulate canonical Wnt signaling, by modulating β-catenin activity. USP4 deubiquitinases TCF4, to suppress β-catenin dependent transcription [143]. While USP15 deubiquitinates and stabilizes tumor suppressor adenomatous polyposis coli (APC) involved in the proteasomal degradation of β-catenin [144]. TRABID (TRAF-binding domain-containing protein) or ZRANB1 (zinc finger Ran-binding domain-containing protein 1) deubiquitinates APC by removing K63-linked Ub chains, inducing TCF4-mediated transcription upon Wnt stimulation [145].

3.4.3. NFκB Signaling. Activation of IKK leads to phosphorylation and degradation of the IκBs, promoting the nuclear translocation of NFκB. K63-linked Ub-NEMO binds IKK recruiting it to complex I, activating NFκB (via cIAP 1 and 2; cFLIP; and TNF-α and IL-8); or JNK and p38 MAPK; for antiapoptotic or proinflammatory responses, respectively, [146]. K63-linked Ub chains on RIP1 serve as a scaffold for binding of TAK1 and TAB2. Upon TNF-α induction, RIP1 ubiquitination may show three specific outcomes. In the first case, K48-linked ubiquitination of RIP1 may lead to targeting it for degradation and triggering the apoptotic cascade. In the second case, K63-linked ubiquitination of RIP1 may lead to association of complex I, inducing proinflammatory or antiapoptotic response. In the third case, removal of K63-linked ubiquitin chains from RIP1 may lead to the association of complex II again sending the cells to death pathway. Hence, it is clear that the ubiquitination of these factors, especially, the ubiquitin linkage of RIP1 is very important in determining cellular fate. The involvement of multiple DUBs in this pathway shows the importance of deubiquitinating enzymes in determining the cell fate via this signaling axis [95].

CYLD. CYLD is a negative regulator of NFκB signaling contributing to oncogenesis. Knockdown of CYLD resulted in enhanced NFκB signaling upon TNF-α stimulation [147]. The members of NFκB signaling are subjected to K63-linked ubiquitination for their activation which is again deubiquitinated, as for example–CYLD deubiquitinates with NEMO, TRAF2, 6 and 7, RIP1 and TAK1 [148–150]. CYLD itself is regulated by NFκB creating a negative feedback loop [151]. CYLD also inactivates BCL-3 by deubiquitination [152]. As BCL-3 is a coactivator of NFκB, this inactivation of BCL-3 leads to switching in the transcriptional activity of NFκB from repression to activation of cell proliferation [153, 154]. CYLD negatively regulates BCL-3 mediated NFκB signaling by induction of p50-BCL3 and p52-BCL3 complexes and inhibiting cell proliferation [152].

A20. A20 negatively regulates NFκB signaling. The targets of A20-mediated deubiquitination are K63-linked ubiquitinated TRAF6 and RIP1 [155, 156]. The enzyme A20 has been shown to have a dual role on RIP1, by acting both as a DUB (removing K63-Ub) as well as an E3 ligase (linking K48-Ub and marking it for degradation), negatively regulating the NFκB signaling [156]. Apo2L induces both K48 and K63-linked polyubiquitination of caspase 8. K63-linked caspase 8 ubiquitination makes it enzymatically more potent leading to apoptosis. A20 acts as a DUB removing the K63-Ub and has been hypothesized to add K48-Ub due to its E3 ligase activity similar to its dual role as seen in case of RIP1 [157]. In B-cell lymphomas A20 is frequently inactivated and upon its restoration, cells undergo apoptosis [158].

USP7. USP7 is ubiquitinated by TRIM27 complex, which deubiquitinates RIP1, resulting in positive regulation of TNF-α induced apoptosis [58]. USP7 deubiquitinates p65-NFκB by interacting with p65 at the target gene promoters, increasing their transcription [159].

Other DUBs. USP2a removes K63-linked Ub chains from RIP1 and TRAF2 again negatively regulating NFκB signaling pathway [160]. Both USP11 and USP15 also negatively regulate and function by deubiquitinating IκB to sequester NFκB in the cytoplasm. USP11 stabilizes IKK-α to stabilize and activate p53 [161]. USP21 and Cezanne or OTUD7B negatively regulate NFκB signaling by deubiquitinating and inactivating RIP1 upon TNF-α stimulation, providing a feedback loop in the proinflammatory signaling [162]. Cezanne has been reported to hydrolyze linear K11-Ub chains from RIP1. MCPIP1 also removes K63-Ub from RIP1 and TRAF2 and 6 but its activity is unclear [163].

3.4.4. Other Oncogenic Signals. USP2 deubiquitinates both Mdm2 and MdmX. Upon androgen stimulation in prostate cancer (PCa) cells, USP2 deubiquitinates and stabilizes Mdm2 [164] and its homologue MdmX [165]. USP2a can also deubiquitinate Fas preventing apoptosis in PCa [166]. cMyc plays a pivotal role in cell growth, differentiation, and apoptosis by regulating (both activating and repressing) the transcription of proteins involved in cell cycle, survival, protein synthesis, cell adhesion, and so forth, depending on its interacting partner in forming the functional heterodimer [167]. cMyc shows multiple mutations in various cancers. The UPS mediated degradation of cMyc involves an array of E3 ligases. USP28, often overexpressed in cancers, was shown to stabilize cMyc by reversing FBW7-α mediated ubiquitination [168].

3.4.5. Tumor Suppressors and Associated Pathways. USP7 deubiquitinates tumor suppressors p53, PTEN, FOXO, Rb, and so forth. The stabilization of p53 is triggered upon DNA damage stress, while under normal conditions Mdm2 is a better target for USP7 [107, 169]. USP7 helps in removing mono-Ub link on PTEN, facilitating its translocation back to the cytosol, which enhances tumor aggressiveness in PCa. USP7 also regulates $p27^{Kip1}$ via deubiquitination of the FOXOs (3a and 4). FOXO 3a and 4 are deubiquitinated by USP7, to be retained in the nucleus to promote their tumor suppressive transcriptional activity [170]. In case of Rb, USP7 shows a differential deubiquitination in normal and cancer (glioma) cells depending on the Mdm2 levels [106]. Regulation of p53 is also subject to other DUBs, such as USP4, USP10, USP29, and USP42 [171]. USP2a deubiquitinates Mdm2 hence promoting p53 degradation [164].

3.5. DUBs as Therapeutic Targets in Cancer. Some of the conventional chemotherapeutic drugs generally rely on restoration of the endogenous cell death pathways such as activation of proapoptotic proteins like Bax, PUMA, caspases, Apaf-1, and so forth; induction of p53 apoptotic pathway by generating genotoxic stress; creating DNA damage to trigger the repair mechanisms; pharmacological inhibitors and monoclonal antibodies to target oncogenic receptors (e.g., EGFR), kinases (e.g., Akt), transcription factors (e.g., Stat3, cMyc, NFκB), and so forth to regulate the major signaling pathways. In the clinical context, differentially targeting cancer cells from normal cells according to the above strategies largely depends on the dependency of cancer cells on these aberrant signals. But cellular signaling redundancy overpowers, leading to resistance. Also many drugs turn out to be carcinogenic as discussed by Apraiz et al. [172]. This can be avoided by targeting the UPS and DUBs. Some of the possibilities are restoration of tumor suppressive deubiquitinases like CYLD [173] and inhibition of oncogenic deubiquitinases like USP9X [139]. But this approach is also not that straight forward because of the dual nature of certain DUBs like USP7.

The UPS controls a large number of cell cycle proteins such as p27, Rb, cyclin D1, p53, the Bcl-2 family members, and NFκB signaling [174, 175]. And as discussed earlier in Section 1, the successful use of bortezomib in multiple myeloma (MM) utilizes the aggregation of misfolded proteins in cancer cells as compared to their normal counterparts. In this cellular perspective, the principles of autophagy, that is, nutrient recycling via delivery of cytosolic contents to the lysosomes, has been found to be utilized in disposing protein aggregates, leading to the emergence of the "quality control autophagy" concept. The two ubiquitin binding proteins p62 and HDAC6 recognize the Ub-linked protein aggregates that have evaded proteasomal degradation and reroute them to the aggresome-autophagy machinery. The DUB ataxin 3 plays an important role here in remodelling the Ub-tags on protein aggregates to modify the existing signals and subsequent cell fate [176]. Combinatorial antitumoral effects of autophagic and proteasomal inhibitors have shown promises in MM [177].

The DUBs themselves are subject to different modes of regulation like transcriptional, post-translational, change in subcellular localization via multiple protein-protein interactions as discussed by Fraile et al. [20]. Some of the regulatory mechanisms are feedback loop (e.g., autoproteolysis of USP1; proteolysis of CYLD and A20), caspase 3 mediated proteolytic processing of USP7 (as reported in playing a role in thymocyte apoptosis [178]), phosphorylation (inactivates CYLD, USP8; activates A20, USP7, USP15, USP19, USP28, USP34, USP37), ubiquitination (inactivates UCH-L1, USP7, USP36), neddylation and sumoylation (inactivates USP25); ROS (modifies Cezanne); targeting of USP30 to mitochondria, or USP36 to nucleoli, and so forth. From the structural point of view, conserved catalytic sites may be blocked or conformational changes may modulate their activity (UCH-L1, OTU1, USP1, USP7, USP12, and USP46).

All these reports suggest a potential of DUBs as therapeutic targets in cancer. Till date several strategies to inhibit the DUB activity have been studied. Here we have discussed the various inhibitors of DUBs and the degree of success achieved with regard to cancer cell death. There are many small molecule inhibitors, natural compounds, and their analogs and quite a few are in preclinical trials (see details in Table 3). Some of the inhibitors are broad spectrum, while there are some which are very specific, targeting only a particular DUB.

Table 3: Drugs targeting the DUBs.

Drug	Target	Outcome	Comments	Reference
		Multiple Targets		
Betulinic acid	Multiple DUBs	Accumulates poly-Ub targets and enhances their degradation via UPS; inhibits PCa cell proliferation and induces apoptosis	Specific in action against cancer cells without affecting normal cells	[5]
Curcusone D	Multiple DUBs	ROS-induced inhibition of DUBs; growth inhibition and apoptosis of multiple myeloma (MM) cells	Shows synergistic effect with Bortezomib in MM	[6]
PR-619	Multiple DUBs	Accumulates polyubiquitinated proteins and enhances proteasomal activity	Small molecule inhibitor	[7]
Cyclopentenone prostaglandins, dibenzylideneacetone, curcumin, and shikoccin	Cellular isopept-idase	Cell death in colon cancer cells	Very broad spectrum	[8]
b-AP15	UCH-L5 USP14	Blocks proteasome function and promotes tumor cell apoptosis in preclinical models	High specificity	[9]
P22077	USP7 USP47	Induction of apoptosis involving p53 by multiple pathways (Mdm2, Claspin, Tip60, etc.)	Specific small molecule inhibitor	[7, 134]
WP1130	USP5 USP9X USP14 UCH-L1 UCH-37	Decreased Mcl-1, increased p53, stops cell proliferation and induces death in multiple myeloma (MM) and mantle cell lymphoma (MCL)	Poor solubility and pharmacokinetics	[10]
G9 (a WP1130 derivative)	USP9X USP24	Decreased Mcl-1, increased p53, induces cell death in MM, MCL and melanoma	Better solubility and lesser toxicity than WP1130	[10]
		Specific Targets		
3-Amino-2-keto-7H-thieno[2,3-b]pyridin-6-one derivatives	UCH-L1	Moderately potent inhibitors	Does not show activity against other cysteine hydrolases	[11]
Pimozide and GW7647	USP1	Shows synergistic effect with cisplatin in cytotoxicity of NSCLC	Specificity is high	[12]
2-cyanopyrimidine and triazine derivatives	USP2	Specific biological data is absent	—	[8]
HBX 41,108	USP7	Activates p53 and induces apoptosis in cancer cells	Shows some cross-reactivity	[13]
HBX 19,818 and HBX 28,258	USP7	Leads to cell cycle arrest in HCT116 cells	Specific inhibition of catalytic activity	[14]
Spongiacidin C	USP7	—	A natural pyrrole alkaloid	[15]
HBX 90,397 and HBX 90,659	USP8	Induces G1 arrest and inhibits cell growth in HCT116 and PC3	Small molecule inhibitor	[16]
9-oxo-9 H-indeno[1,2-b]pyrazine-2,3-dicarbonitrile and analogs	USP8	Antiproliferative and proapoptotic in cancer cell lines	Selective inhibitor	[17]
1-[1-(4-fluorophenyl)-2,5-dimethylpyrrol-3-yl]-2-pyrrolidin-1-ylethanone)-IU1	USP14	Enhances proteasome function and accelerates proteolysis	Small molecule inhibitor	[18]

On the basis of the wide range of DUB substrates, it is very interesting to target them to induce cell death pathways in the cancer cells. But due to the highly context-dependent functioning of these DUBs, it becomes very important to understand the detailed molecular mechanism of the DUB activity in a specific pathway before targeting them with inhibitors. All the processes discussed in Section 3 can be exploited to our benefit. It may be speculated that this approach of targeting DUBs may have an edge over the conventional therapies to combat the tendency of cancer cells to acquire resistance. It is also suggestive of a combinatorial drug treatment of DUB inhibitors with the conventional cytotoxic drugs [171].

4. Concluding Remarks

It is well established that uncontrolled cell growth in cancer is not an outcome of abnormal cell proliferation alone but is also because of the lack of cell death, especially, the pathways that are programmed. The significance of cell death pathways in the perspective of cancer is that any deregulation in cell death leads to abnormal cell survival and proliferation, leading to oncogenesis. Hence, the knowledge of the various signaling pathways involved in cell death and their crosstalks is very important to figure out the most plausible therapeutic targets. During radio- or chemotherapy, one of the strategies exploited is the reactivation of signaling pathways, to induce cell death. The major hurdle in cancer treatment has been the redundancy in signaling pathways, favoring development of resistance against conventional therapies [179]. Hence, the requirement of focusing on alternative strategies arises.

Abbreviations

AIF: Apoptosis inducing factor
Apaf-1: Apoptotic protease activating factor 1
Cdk: Cyclin-dependent kinase
Chk: Checkpoint kinase
DISC: Death-inducing signaling complex
DUB: Deubiquitinating enzyme or deubiquitinase
Endo G: Endonuclease G
ER: Endoplasmic reticulum
FADD: Fas associated death domain
FOXO: Forkhead box protein
IAP: Inhibitor of apoptosis
JAMM: JAMM/MPN domain-associated metallopeptidase
K: Lysine
MAPK: Mitogen-activated protein kinase
MCPIP: Monocyte chemotactic protein-induced protease
MJD: Machado–Joseph disease protein domain protease
MOMP: Mitochondrial outer membrane permeability
OTU: Ovarian-tumor protease
PARP: Poly-ADP-ribose-polymerase
Pol: Polymerase
PTEN: Phosphatase and tensin homolog
RIP: Receptor interacting protein kinase
ROS: Reactive oxygen species
RTK: Receptor tyrosine kinase
TNF: Tumor necrosis factor
TNFR: TNF receptor
TRADD: TNF receptor associated death domain
TRAIL: TNF Apoptosis-Inducing Ligand
Ub: Ubiquitin
UCH: Ubiquitin carboxy-terminal hydrolase
UPS: Ubiquitin proteasome system
USP: Ubiquitin specific protease.

Conflict of Interests

The authors declare no conflict of interests.

Authors' Contribution

Mrinal Kanti Ghosh and Seemana Bhattacharya wrote and analyzed the paper.

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Research Article

Necroptosis Mediates TNF-Induced Toxicity of Hippocampal Neurons

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Tumor necrosis factor-α (TNF-α) is a critical proinflammatory cytokine regulating neuroinflammation. Elevated levels of TNF-α have been associated with various neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease. However, the signaling events that lead to TNF-α-initiated neurotoxicity are still unclear. Here, we report that RIP3-mediated necroptosis, a form of regulated necrosis, is activated in the mouse hippocampus after intracerebroventricular injection of TNF-α. RIP3 deficiency attenuates TNF-α-initiated loss of hippocampal neurons. Furthermore, we characterized the molecular mechanism of TNF-α-induced neurotoxicity in HT-22 hippocampal neuronal cells. HT-22 cells are sensitive to TNF-α only upon caspase blockage and subsequently undergo necrosis. The cell death is suppressed by knockdown of CYLD or RIP1 or RIP3 or MLKL, suggesting that this necrosis is necroptosis and mediated by CYLD-RIP1-RIP3-MLKL signaling pathway. TNF-α-induced necroptosis of HT-22 cells is largely independent of both ROS accumulation and calcium influx although these events have been shown to be critical for necroptosis in certain cell lines. Taken together, these data not only provide the first *in vivo* evidence for a role of RIP3 in TNF-α-induced toxicity of hippocampal neurons, but also demonstrate that TNF-α promotes CYLD-RIP1-RIP3-MLKL-mediated necroptosis of hippocampal neurons largely bypassing ROS accumulation and calcium influx.

1. Introduction

Massive loss of a particular subset of neurons is a pathological hallmark of neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), and multiple sclerosis (MS). Cytokine-driven neuroinflammation and neurotoxicity have been implicated in the initiation and progression of these devastating diseases [1]. Ample evidence suggests that tumor necrosis factor-α (TNF-α) is a key proinflammatory cytokine regulating neuroinflammation and plays roles in both homeostasis and disease pathophysiology in the central nervous system (CNS) [2]. TNF-α is commonly elevated in the clinic and animal models of neurodegenerative diseases. For example, increased level of TNF-α is detected in the brain and plasma in AD patients and mouse models of AD. In CNS, TNF-α is mainly produced by activated microglia and astrocytes in response to various stimuli including infection and injury. Genetic deletion of TNFR1 has been shown to attenuate the production of the amyloid-β (Aβ) and to improve impairments in mice with AD [3, 4]. Moreover, deficiency of TNF-α or TNF receptor protects against dopaminergic neurotoxicity [5, 6]. Therefore, overproduction of TNF-α is strongly linked with neuronal damage, and blockage of TNF-α-mediated neurotoxic pathway emerges as an attractive strategy for the treatment of degenerative diseases such as AD and PD. Although TNF-α has been shown to be neurotoxic to cultured neurons by promoting glutamate production [7], the signaling events that lead to TNF-α-initiated neurotoxicity are not yet understood.

As a pleiotropic factor, TNF-α is involved in diverse cellular responses including apoptosis and necrosis. TNF family of cytokines, such as TNF-α, TRAIL, and FasL, triggers apoptosis by recruiting and activating caspase-8 through the adaptor protein FADD. In some cell types, suppression of

caspase-8 or FADD sensitizes cells to programmed necrosis (termed necroptosis) in response to these cytokines as well as ligands of Toll-like receptors (TLRs) [8, 9]. Necroptosis depends on the formation of a necrosome complex, which contains receptor-interacting kinase-1 (RIP1) [10], receptor-interacting kinase-3 (RIP3) [11–13], and mixed lineage kinase domain-like protein (MLKL) [14, 15]. In TNF-α-induced necroptosis, deubiquitination of RIP1 by cylindromatosis (CYLD) is a critical process for necrosome formation and activation [16, 17]. Although downstream mechanisms mediating execution of necroptosis remain to be elucidated, reactive oxygen species (ROS) accumulation [13, 18] and calcium influx [19] have been shown to be critical for necroptosis in certain cell lines.

The connection between necroptosis and neuronal damage has been suggested by studies demonstrating a protective effect of necroptosis inhibitor on brain injury in experimental stroke and trauma models [20, 21]. We therefore hypothesize that necroptosis is activated during neuroinflammation and further drives neurotoxicity. To this end, we used RIP3-deficient mice to determine the regulation of necroptosis in TNF-α-induced neurotoxicity *in vivo*. Here, we demonstrated that deficiency of RIP3 alleviates the loss of hippocampal neurons in the mouse hippocampus after intracerebroventricular injection of TNF-α. Using an *in vitro* hippocampal neuronal model, we provided a detailed molecular characterization of TNF-α-induced death of hippocampal neurons.

2. Materials and Methods

2.1. Animal Models. RIP3 knockout mice were generated as described previously [11] and crossed to C57BL/6 mice for ten generations. Female wild-type and RIP3 knockout mice at 6–8 weeks of age received intracerebroventricular injection of TNF-α. In brief, 2.5 μg or 5 μg TNF-α was dissolved in PBS to make a total volume of 20 μL and then injected into each lateral ventricle. The control group mice received 20 μL PBS. After 3 days, mice were scarified and the proteins were extracted from hippocampus and subjected to western blot analysis. Morphology of hippocampal neurons was analyzed by Nissl staining of brain sections. All animal experiments were performed in accordance with protocols by the Institutional Animal Care and Use Committee at Soochow University.

2.2. Reagents. Dulbecco's modified Eagle's medium (DMEM) was from Thermo. Penicillin/streptomycin, L-Glutamine, and fetal bovine serum (FBS) were from GIBCO. BHA, NAC, phosphate buffered saline (PBS), and Lanthanum(III) chloride heptahydrate ($LaCl_3{\cdot}7H_2O$) were from Sigma. Recombinant TNF-α was purified as described previously [11]. z-VAD was from Bachem. Necrostatin-1 was from Alexis Biochemicals. Propidium Iodide was from Biouniquer. The following antibodies were used for western blotting: mouse RIP3 (Prosci, 2283), RIP1 (BD Biosciences, 610459), mouse CYLD (Cell Signaling, 437700), caspase-3 (Cell Signaling, 9662), and β-actin (Sigma).

2.3. Cell Culture. Mouse hippocampal neuron (HT-22) cells were a gift from the Lab of Dr. Zhenghong Qin (Soochow University). Mouse embryonic fibroblasts (MEFs) were isolated from day 14.5–15.5 embryos. These cells were grown in DMEM supplemented with 10% fetal bovine serum.

2.4. Plasmids and Oligos. Lentiviral expression construct containing mouse RIP3 was amplified from RIP3 plasmid with primers containing an N-terminal Flag epitope and then cloned into pCAG-MCS-IRES vector that was a gift from the Lab of Dr. Yun Zhao (Soochow University). Lentiviral expression construct containing RIP3-RHIM domain mutant (RIP3-RHIM-Mut) or RIP3 kinase mutant (RIP3 K51A) was generated by QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies). Mouse RIP3, RIP1, MLKL, and CYLD siRNAs were synthesized by GenePharma: RIP3-1 (cccgacgaugucuucugucaa), RIP3-2 (cuccuuaaagucaauaaacau), RIP1-1 (ccacuagucugacugauga), RIP1-2 (ucaccaauguugcaggaua), CYLD-1 (uccauugaggauguaaauaaa), CYLD-2 (aaggguugaaccauuguuaaa), MLKL-1 (gagauccaguucaacgaua), and MLKL-2 (uaccaucaaaguauucaacaa).

2.5. Nissl Staining. The mice were sacrificed 72 h after intracerebroventricular injection of TNFα. Brains were dissected out of the skull and put in 4% paraformaldehyde to fix the tissue for 24 hours at room temperature and then stored in 30% sucrose phosphate buffer overnight until the tissue sank to the bottom of the solution. 20 μm sections were cut in the coronal plane using a freezing microtome (Leica CM19500) and mounted on gelatin coated slides. The sections were further stained in 0.1% cresyl violet solution (Sigma-Aldrich) at 37°C for several minutes. Rinse quickly in distilled water followed by differentiation in 95% ethyl alcohol and check microscopically for best result. Dehydrate in 100% alcohol and clear in xylene. Finally, the sections were mounted using a neutral balsam and photos were taken under microscope.

2.6. Western Blot Analysis. Cell pellets were lysed in lysis buffer containing 20 mM Tris-Hcl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 1% Glycerol, 0.5 mM DTT, 1 mM Na3VO4, 25 mM β-glycerol-phosphate, and 1 mM PMSF supplemented with protease inhibitor cocktail (Roche). The mouse tissue was grinded and resuspended in lysis buffer with 0.1% SDS. The resuspended cell pellet or tissue was vortexed for 10 seconds, then incubated on ice for 20 min, and then centrifuged at 20,000 g for 20 min. Protein concentration was determined by Quick Start Bradford 1x Dye Reagent (Bio-Rad). The protein samples were prepared for western blot analysis.

2.7. Generation of Stable Cell Lines. 293T cells werecotransfected with lentiviral expression construct and packaging plasmids mix, and viral particles were collected after 48 hours and 72 hours. HT-22 cells were infected with lentivirus containing RIP3, RIP3K51A, and RIP3-RHIM-Mut, respectively. 72 hours later cells were selected with GFP by fluorescence-activated cell sorting.

2.8. Transfection and Cell Viability Assay. HT-22 cells were transfected with siRNAs by Lipofectamine RNAiMAX

Reagent (Invitrogen) for 60 h and then treated with the indicated drug for about 20 h. Cell survival was determined by Cell Titer-Glo Luminescent Cell Viability Assay kit (Promega).

3. Results

3.1. The Regulation of RIP3 in TNF-α-Induced Toxicity of Hippocampal Neurons In Vivo. RIP3 is a key molecule regulating necroptosis induced by TNF family cytokines and ligands of TLR3/4. Elevated expression of RIP3 protein is observed in the damage tissues and correlates with active necroptosis during the pathogenesis of diseases such as acute pancreatitis, retinal detachment, and liver injury [11, 22, 23]. To assess the role of necroptosis in TNF-α-induced neurotoxicity, we challenged wild-type and RIP3-deficient mice with intracerebroventricular injection of TNF-α. Histological analysis with Nissl staining of neurons was performed to evaluate TNF-α-induced damage of neurons. We observed that administration of TNF-α to wild-type mice caused a reduction in neuronal density in the hippocampus especially CA3 region in a dose-dependent manner as compared with control-treated mice (Figure 1(a)). Notably, no obvious loss of hippocampal neurons was observed in RIP3-deficient mice after treatment of TNF-α (Figure 1(a)). Moreover, we noticed that the expression levels of RIP1 and RIP3 were increased in the hippocampus after TNF-α treatment (Figure 1(b)), while there is no detectable activation of caspase-3 which is an executioner caspase activated via proteolytic cleavage during apoptosis (Figure 1(c)), indicating that necroptosis but not apoptosis is activated by the injection of TNF-α. These results indicate that necroptosis is activated in CNS and contributes to the toxicity of hippocampal neurons in response to TNF-α.

3.2. HT-22 Hippocampal Neurons Are Committed to Necrosis rather than Apoptosis in Response to TNF-α. Having observed RIP3-mediated necroptosis in TNF-α-induced toxicity of hippocampal neurons *in vivo*, we sought to clarify the molecule mechanism underling TNF-α-induced neurotoxicity in HT-22 hippocampal neuronal cell line, which is often employed as an *in vitro* model of hippocampal neuron. We observed that HT-22 cells were resistant to TNF-α, even in the presence of Smac mimetic, a compound which can mimic the function of proapoptotic protein Smac/Diablo and induces apoptosis as a single agent or in combination with TNF-α [24, 25] (Figure 2(a)). Notably, addition of caspase inhibitor, z-VAD, sensitized HT-22 cells to death in response to TNF-α in a dose-dependent manner (Figure 2(a)). Propidium iodide (PI) positive cells were detected in TNF/z-VAD treated HT-22 cells (Figure 2(b)), suggesting that these cells lost membrane permeability and underwent necrosis. Taken together, these data demonstrate that HT-22 hippocampal neuronal cells are committed to TNF-α-induced necrosis rather than apoptosis.

3.3. TNF-α-Induced Necrosis of HT-22 Cells Is Mediated by CYLD-RIP1-RIP3-MLKL Signaling Pathway. RIP3 kinase is a key determinant for necroptosis. RIP3 protein contains an N-terminal serine/threonine kinase domain and a C-terminal RIP homotypic interaction motif (RHIM). The kinase activity and RHIM domain of RIP3 are critical for its function in mediating necroptosis [11]. We examined the role of RIP3 in TNF-α-induced necrosis in HT-22 cells by RNAi approach. Knockdown of endogenous RIP3 greatly blocked TNF-α-induced necrosis (Figures 3(a) and 3(b)), which was restored by stable expression of a shRNA-resistant wild-type RIP3, but not a shRNA-resistant kinase dead form or RHIM mutant form of RIP3 (Figures 3(c) and 3(d)), indicating that both kinase activity and RHIM domain of RIP3 are crucial for TNF-α-induced necrosis of HT-22 cells.

RHIM domain of RIP3 is known to be critical for its interaction with RIP1 during necroptosis [26]. We further tested whether RIP1 is required for TNF-α-induced necrosis of HT-22 cells. As shown in Figures 4(a) and 4(b), reducing endogenous RIP1 suppressed TNF-α-induced necrosis. In addition, knockdown of CYLD, a deubiquitinase of RIP1, blocked TNF-α-induced necrosis of HT-22 cells (Figures 4(c) and 4(d)).

MLKL is a kinase-like protein and functions as a substrate of RIP3. To assess the requirement of MLKL in TNF-α-induced necrosis of HT-22 cells, we performed MLKL RNAi in the cells and found knockdown of MLKL efficiently reduced the cell death (Figures 5(a) and 5(b)).

3.4. TNF-α-Induced Necroptosis of HT-22 Cells Is Largely Independent of ROS Accumulation and Calcium Influx. We and others have shown that ROS accumulation is required for RIP3-mediated necrosis in certain cell lines such as mouse embryonic fibroblast (MEF) [13, 18], so we evaluated the role of ROS in TNF-α-induced necroptosis of HT-22 cells by using two widely used ROS scavengers, butylated hydroxyanisole (BHA) and N-acetylcysteine (NAC). MEF cells are known to undergo necroptosis in response to TNF-α, Smac mimetic and z-VAD. In the presence of BHA at 100 μM, the survival rate of HT-22 cells was increased by 13% and around 30% cells still underwent necrosis in response to TNF-α plus z-VAD, while TNF-α-induced necrosis in MEF cells was entirely prevented by BHA (Figures 6(a) and 6(b)). NAC had no inhibitory effect on TNF-α-induced necrosis of HT-22 cells, whereas the survival rate of MEF cells treated with necroptotic stimuli was increased by 40% after the addition of NAC at 10 mM (Figures 6(a) and 6(b)). Recently, calcium influx has been reported to be essential for necroptosis. We tested whether calcium influx is involved in TNF-α-induced necroptosis of HT-22. As shown in Figure 6(c), inhibition of calcium influx by the addition of $LaCl_3$, a non-voltage-sensitive channel blocker, increased the survival rate of HT-22 by around 14%. Notably, TNF-α-induced necroptosis of HT-22 cells largely proceeded even in the presence of both $LaCl_3$ and NAC (Figure 6(c)). These results demonstrated that TNF-α-initiated necroptosis in HT-22 cells is largely independent of ROS accumulation and calcium influx.

4. Discussion

TNF-α is a key mediator of neuroinflammation. Elevated levels of TNF-α are associated with various neurodegenerative conditions and contribute to neurotoxicity. The mechanisms underlying TNF-α-initiated neurotoxicity are largely

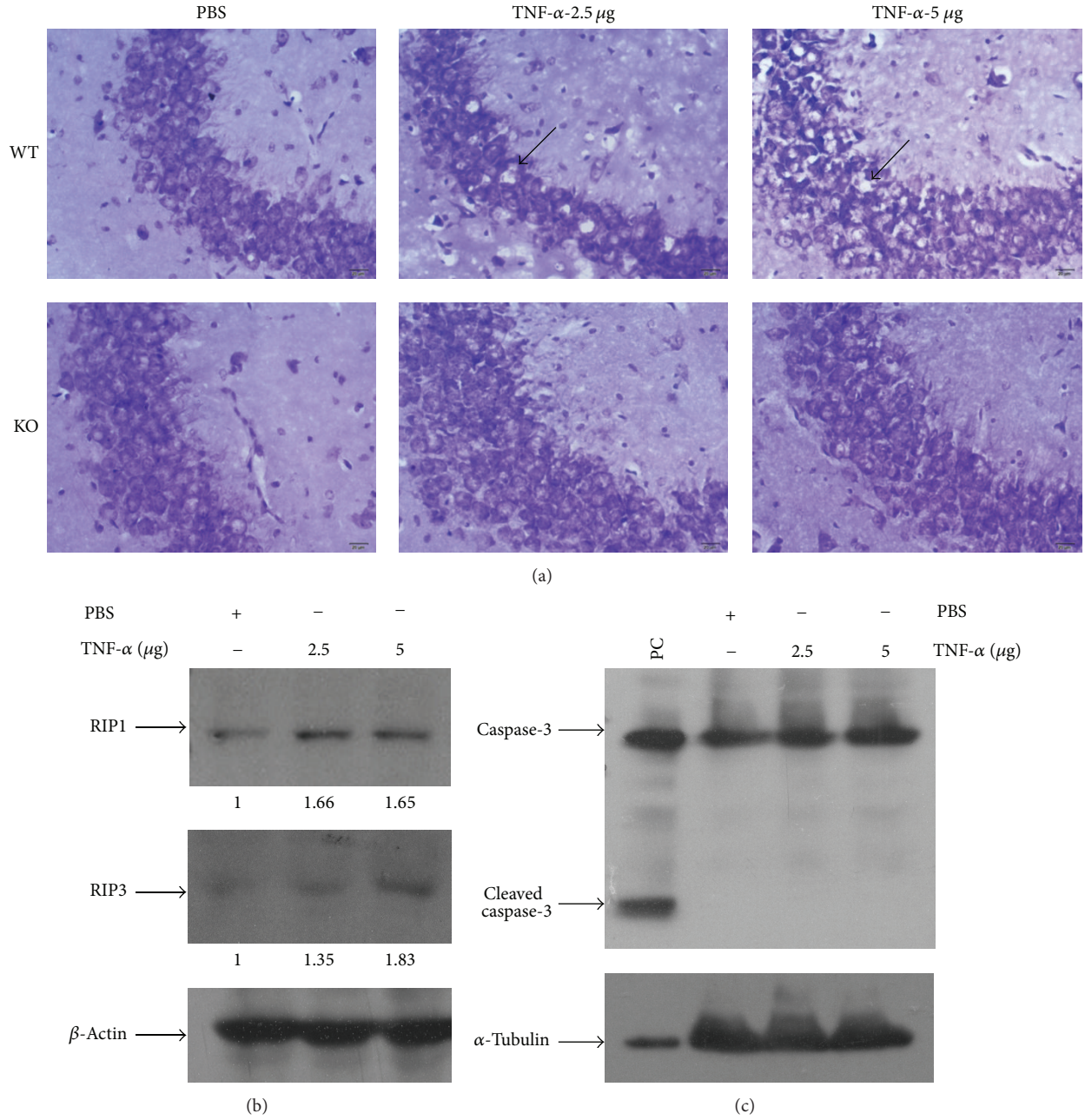

Figure 1: The regulation of RIP3 in TNF-α-induced toxicity of hippocampal neurons *in vivo*. (a) Nissl staining of hippocampal neurons 72 h after treatment. Wild-type (WT) and RIP3 knockout (KO) mice received intracerebroventricular injection of PBS or the indicated dose of TNF-α. The neurons of brain sections from WT and KO mice were analyzed by Nissl staining ($n = 7$) and morphology of hippocampal CA3 region was shown. Arrows indicate the loss of hippocampal neurons. (b) and (c) Expressions of RIP1, RIP3, and caspase-3 in the hippocampus after TNF-α treatment. Proteins extracted from hippocampus in the wild-type mice treated with PBS or TNF-α were analyzed by western blot using indicated antibodies. PC: MEF cells were treated with staurosporine at 150 nM for 15 hours. The results shown here are representative of five mice.

unknown. Our present work revealed an important role of necroptosis in TNF-α-induced toxicity of hippocampal neurons.

Necroptosis is a form of programmed necrosis which is regulated by RIP1 and RIP3 kinases. Apoptosis is known to negatively regulate necroptosis via active caspase-8, which is able to cleave necrosis regulators including RIP1, RIP3, and CYLD [8]. A recent work has shown that mutating the caspase-8 cleavage site at Asp 215 of CYLD is sufficient to promote necroptosis even in the absence of caspase inhibitor, indicating that caspase-8 prevents necroptosis through processing CYLD [27]. CYLD was originally thought to deubiquitinate RIP1 at the membrane receptor complex and promote the recruitment of RIP1 into the necrosome.

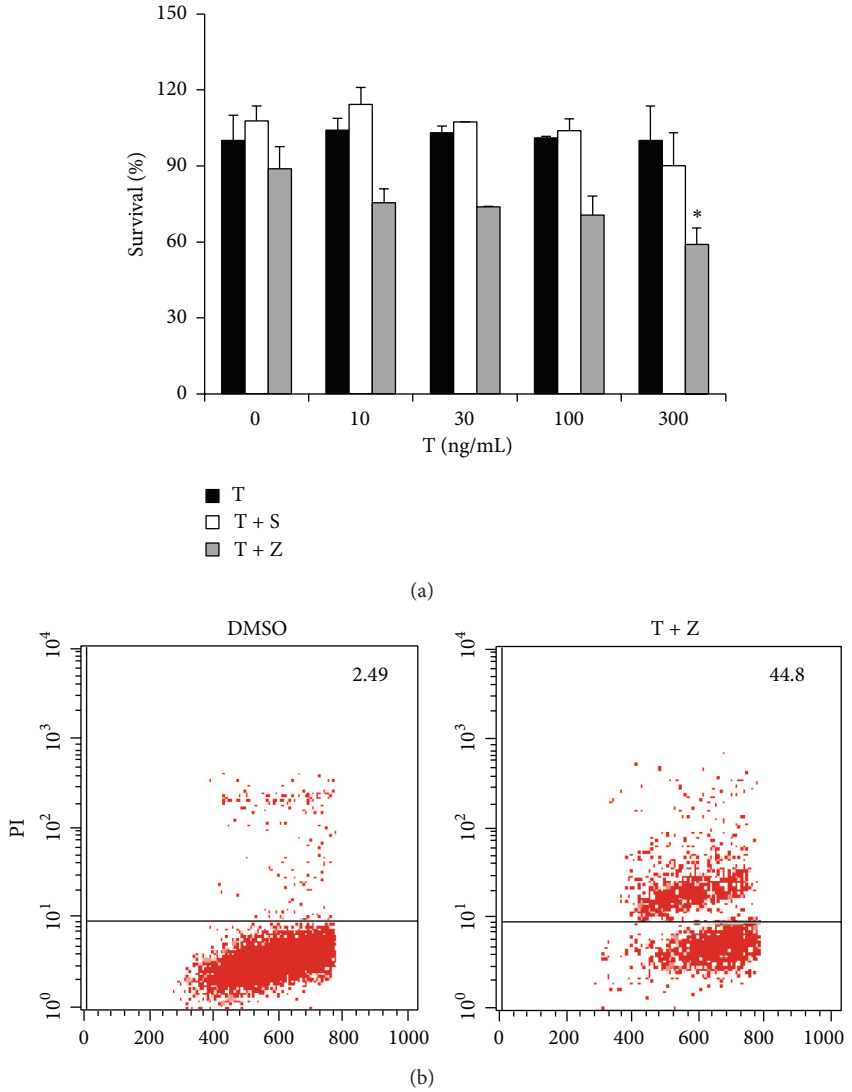

Figure 2: HT-22 hippocampal neurons are committed to necrosis rather than apoptosis in response to TNF-α. (a) HT-22 hippocampal neuronal cells were treated as indicated for 20 h. Cell viability was determined by measuring ATP levels. Data are represented as mean ± standard deviation of duplicates. T: TNF-α; S: Smac mimetic (100 nM); and Z: z-VAD (20μM). (b) HT-22 hippocampal neurons were treated with DMSO or TNF-α (300 ng/mL)/z-VAD for 20 h and then analyzed for PI staining by flow cytometry. Identical concentrations were used in later experiments. Data are represented as mean ± standard deviation of duplicates. $^*P < 0.01$, $^{**}P < 0.001$ versus control. All experiments were repeated three times with similar results.

Recently, it was shown to control the deubiquitination of RIP1 and it facilitates kinase activation in the necrosome [17]. Among the components of necrosome, MLKL is a functional substrate of RIP3. Upon the phosphorylation of MLKL by RIP3, MLKL forms oligomers and locates to the cell plasma membrane [19, 28, 29]. Although mechanisms of necroptosis are extensively studied, there is little known about the machinery of necroptosis in neuronal cells. In this study, we found that HT-22 hippocampal neurons are committed to necroptosis rather than apoptosis in response to TNF-α. Using RNAi approach, we demonstrated that TNF-α-induced neuronal necrosis is mediated by CYLD-RIP1-RIP3-MLKL signaling pathway. Our present study suggests that the core components of necrosome have conserved roles in neuronal necrosis initiated by TNF-α.

Necroptotic cell death is characterized by disrupted plasma membrane; however, the downstream events of necrosome leading to collapse of membrane are largely unknown. ROS production has been shown to be required for necroptosis in several mouse cell lines including L929, MEF, NIH3T3, and macrophages, whereas it is not involved in necroptosis in HT-29 human colon cancer cells [30]. NADPH oxidase NOX1 and metabolic enzymes have been implicated in the control of ROS production during necroptosis [13, 31]. We have shown that TNF-α-induced necroptosis of HT-22 cells mouse hippocampal neurons is largely independent of ROS accumulation. Recently, TRPM7 has been identified to mediate calcium influx through acting downstream of MLKL membrane localization in TNF-α-induced necroptosis [28]. In our study, we showed that TNF-α-induced necroptosis

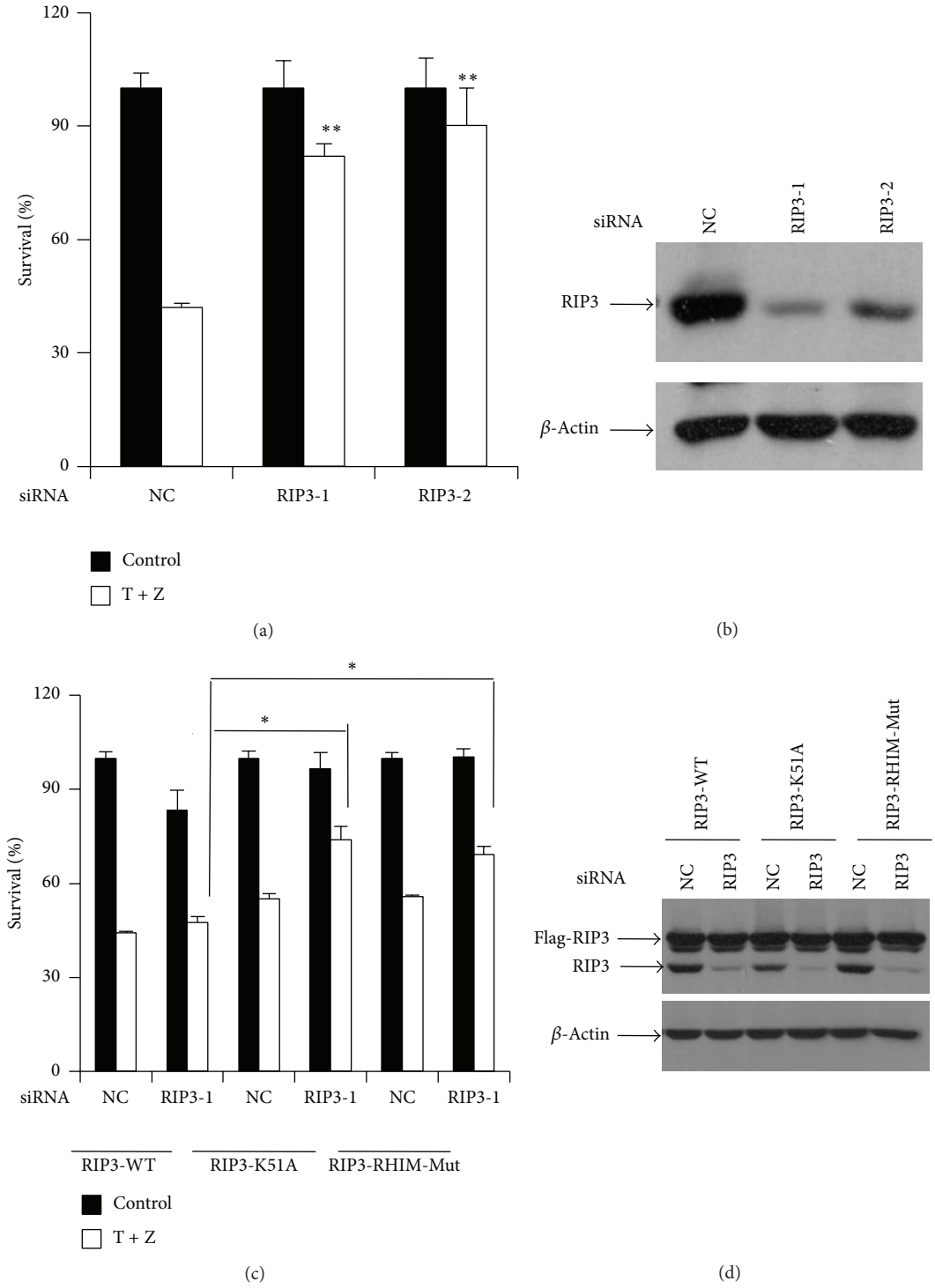

Figure 3: TNF-α-induced necrosis of HT-22 cells depends on RIP3 and its kinase activity. (a) HT-22 cells were transfected with the negative control (NC) or RIP3 siRNAs. After 60 h, cells were treated with control or TNF-α/z-VAD for another 20 h and then cell viability was determined by measuring ATP levels. Data were represented as mean ± standard deviation of duplicates. $^{*}P < 0.01$, $^{**}P < 0.001$ versus NC-T + Z. (b) The knockdown efficiency of RIP3 RNAi. Cell lysates were collected 60 h after transfection and subjected to western blot analysis of RIP3 and β-actin levels. (c) HT-22 cells stably expressing a siRNA-resistant WT-RIP3 or RIP3-K51A or RIP3-RHIM-Mut were transfected with the control or RIP3 siRNAs. After 60 h, cells were treated with control or TNF-α/z-VAD for 20 h and then cell viability was determined by measuring ATP levels. Data were represented as mean ± standard deviation of duplicates. $^{*}P < 0.01$, $^{**}P < 0.001$ versus NC-T + Z. WT-RIP3: HT-22 cells stably expressing a siRNA-resistant wild-type form of RIP3; RIP3-K51A: HT-22 cells stably expressing a siRNA-resistant RIP3 kinase dead mutant. RIP3-RHIM Mut: HT-22 cells stably expressing a siRNA-resistant RHIM domain mutant form of RIP3. (d) The knockdown efficiency of RIP3 RNAi. Cell lysates were collected 60 h after transfection and subjected to western blot analysis of RIP3 and β-actin levels. All experiments were repeated three times with similar results.

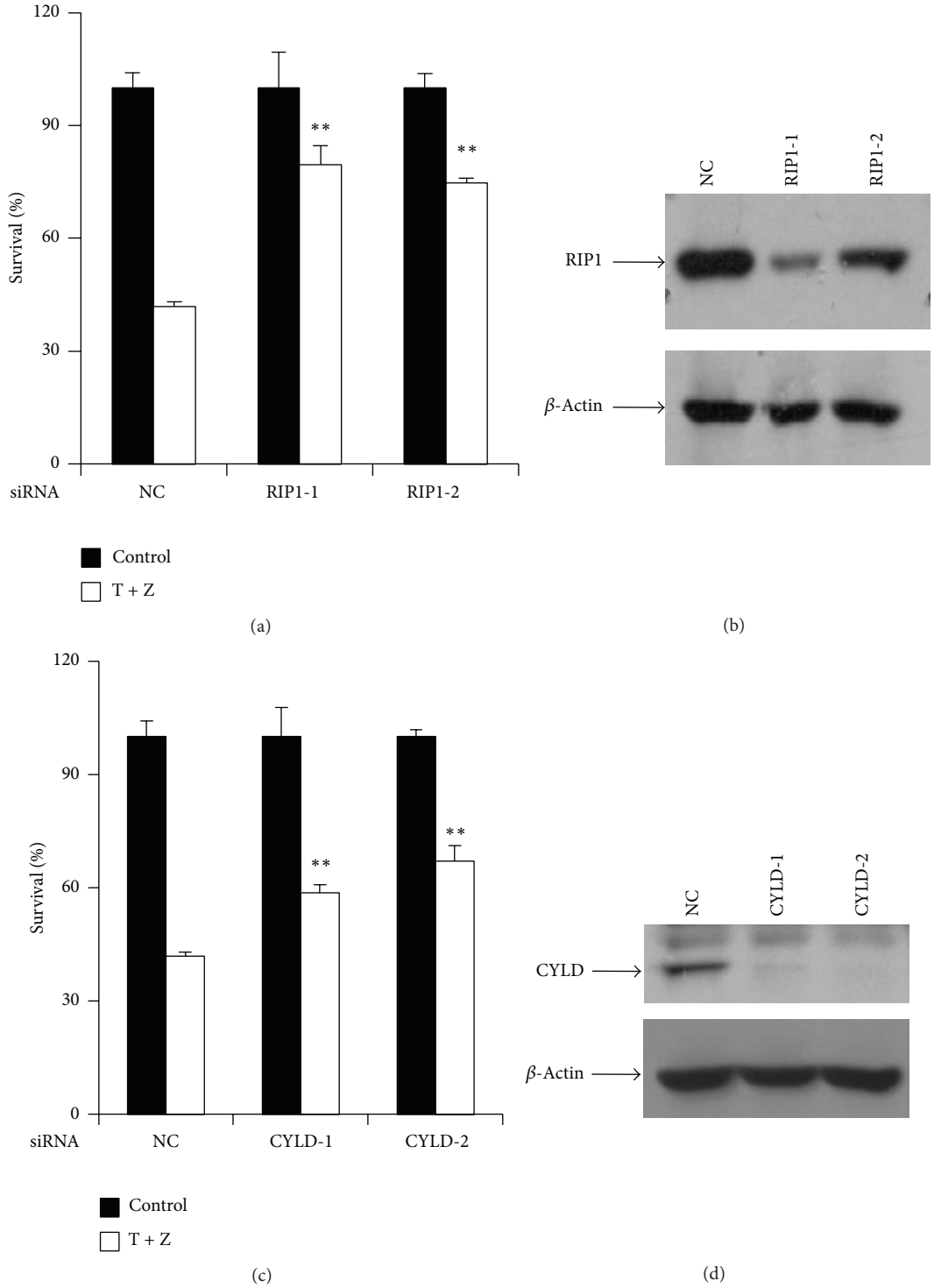

Figure 4: RIP1 and its deubiquitinase CYLD are required for TNF-α-induced necrosis of HT-22 cells. (a) HT-22 cells were transfected with the negative control or RIP1 siRNAs. After 60 h, cells were treated with control or TNF-α/z-VAD for another 20 h and then cell viability was determined by measuring ATP levels. Data were represented as mean ± standard deviation of duplicates. $^{*}P < 0.01$, $^{**}P < 0.001$ versus NC-T + Z. (b) The knockdown efficiency of RIP1 RNAi. Cell lysates were collected 60 h after transfection and subjected to western blot analysis of RIP1 and β-actin levels. (c) HT-22 cells were transfected with the negative control or CYLD siRNAs. Forty-eight hours after transfection, cells were treated with control or TNF-α/z-VAD for another 20 h and then cell viability was determined by measuring ATP levels. Data were represented as mean ± standard deviation of duplicates. $^{*}P < 0.01$, $^{**}P < 0.001$ versus NC-T + Z. (d) The knockdown efficiency of CYLD RNAi. Cell lysates were collected 60 h after transfection and subjected to western blot analysis of CYLD and β-actin levels. All experiments were repeated three times with similar results.

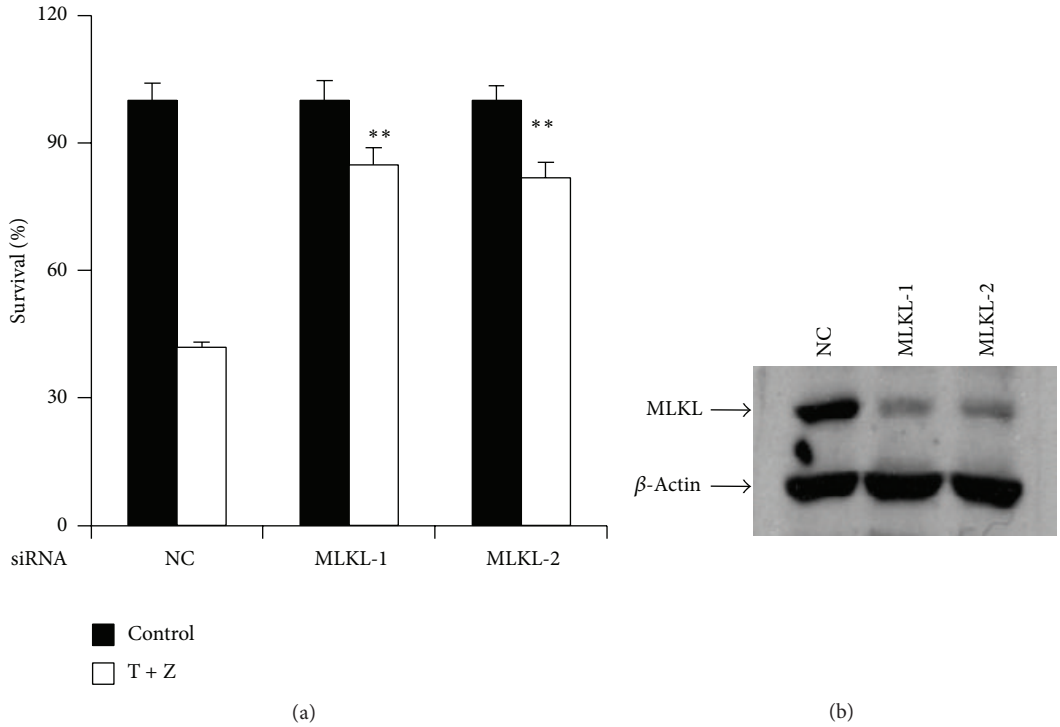

Figure 5: MLKL is essential for TNF-α-induced necrosis of HT-22 cells. (a) HT-22 cells were transfected with the negative control or MLKL siRNAs. After 60 h, cells were treated with control or TNF-α/z-VAD for another 20 h and then cell viability was determined by measuring ATP levels. Data were represented as mean ± standard deviation of duplicates. $^{*}P < 0.01$, $^{**}P < 0.001$ versus NC-T + Z. (b) The knockdown efficiency of MLKL RNAi. Cell lysates were collected 60 h after transfection and subjected to western blot analysis of MLKL and β-actin levels. All experiments were repeated three times with similar results.

of HT-22 cells largely proceeds even in the presence of calcium channel inhibitor and ROS scavenger. The data indicate that TNF-α-induced necroptosis of HT-22 cells largely bypasses ROS accumulation and calcium influx. Therefore, it is tempting to speculate that different downstream responses activated by necrosome depend on cell type. Future studies are required to clarify crucial downstream events for the execution of necroptosis in hippocampal neurons.

Necroptosis is emerging as an important process involved in various pathological conditions including ischemic injury, acute pancreatitis, inflammatory bowel disease, and neuronal damage. Recent studies have demonstrated that increased level of RIP3 expression in the damage tissue correlates with the induction of necroptosis in the mouse models of acute pancreatitis and inflammatory bowel disease (IBD) [30] and patients with IBD [32]. Interestingly, we observed elevated expressions of RIP3 and RIP1 proteins and neuronal cell death in the hippocampus after intracerebroventricular injection of TNF-α. Importantly, genetic deletion of RIP3 reduced the loss of hippocampal neurons after intracerebroventricular injection of TNF-α. To our knowledge, the data represent the first *in vivo* evidence for a role of RIP3 in TNF-α-induced neurotoxicity of hippocampal neurons. Blockage of necroptosis by necrostatin-1, a chemical inhibitor targeting RIP1 kinase, has provided protective effects on neuronal damage in animal models of brain injury [20], stroke [18], and amyotrophic lateral sclerosis [33]. Our study has demonstrated that necroptosis mediates TNF-α-initiated damage of hippocampal neurons. Given the elevated levels of TNF-α in the brains during various neurodegenerative diseases, neuronal cells may be susceptible to necroptosis upon stimulation of TNF-α, therefore contributing to the pathogenesis of neurodegenerative diseases. Targeted prevention of neuronal necroptosis may provide a novel therapeutic approach for the treatment of the related neurodegenerative diseases.

5. Conclusions

Taken together, our study revealed an important role of necroptosis in TNF-α-induced neurotoxicity. Necroptosis can be activated in the mouse hippocampus after intracerebroventricular injection of TNF-α. RIP3 deficiency attenuates TNF-α-initiated loss of hippocampal neurons. HT-22 hippocampal cells are sensitive to TNF-α only upon caspase blockage and subsequently undergo necrosis. A detailed molecular characterization demonstrates that TNF-α-induced necrosis in HT-22 cells is mediated by CYLD-RIP1-RIP3-MLKL necroptotic signaling pathway and largely independent of both ROS accumulation and calcium influx.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

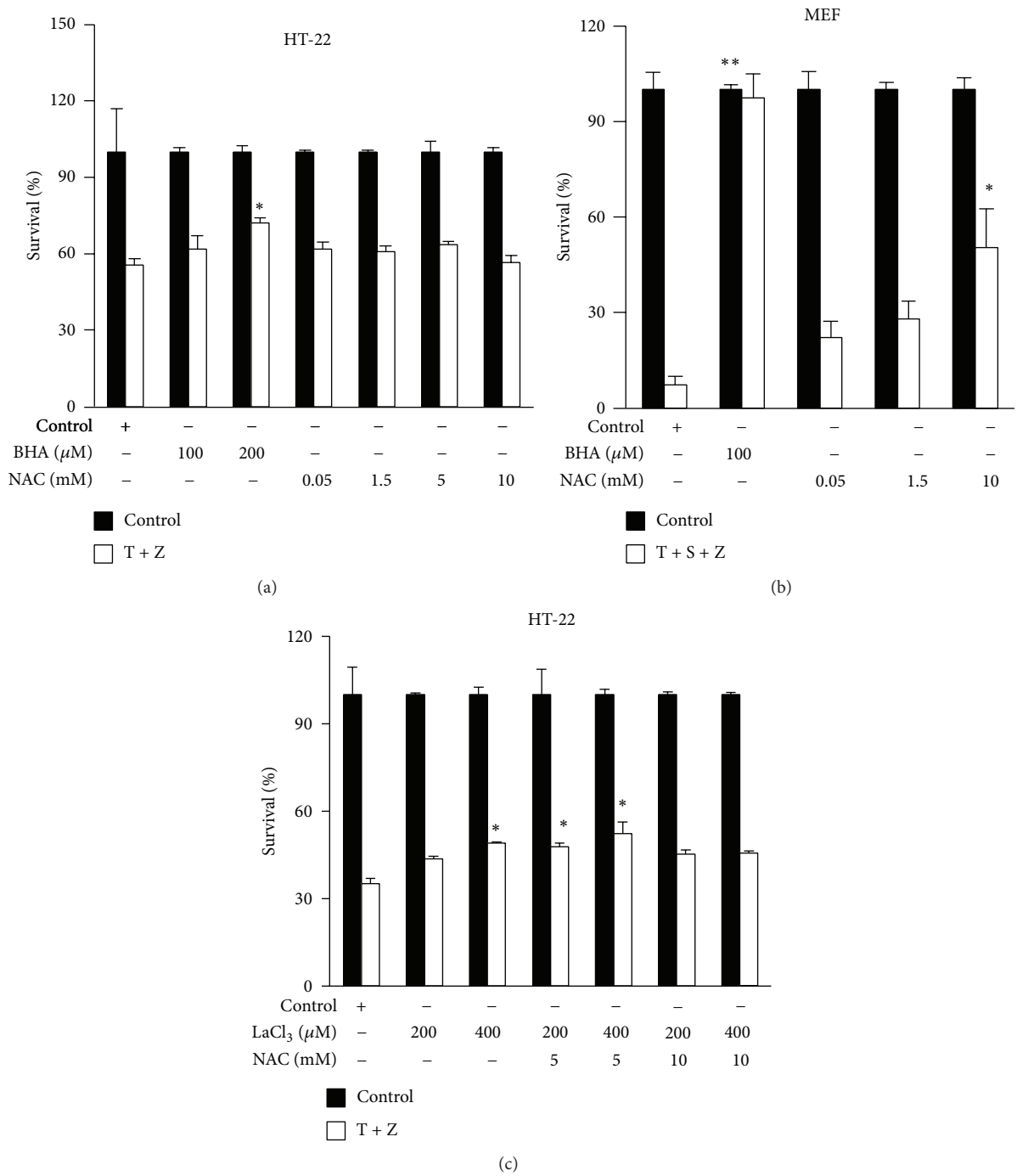

FIGURE 6: TNF-α-induced necrosis of HT-22 cells is largely independent of ROS accumulation and calcium. (a) HT-22 cells were treated with control or BHA or NAC at the indicated concentration 3 h before the treatment of control or TNF-α/z-VAD for 20 h; cell viability was determined by measuring ATP levels. $^{*}P < 0.01$, $^{**}P < 0.001$ versus control-T + Z. (b) MEF cells were treated with control or BMS NAC at the indicated concentration 3 h before the treatment of control or TNF-α/Smac mimetic/z-VAD for 20 h; cell viability was determined by measuring ATP levels. $^{*}P < 0.01$, $^{**}P < 0.001$ versus control-T + S + Z. (c) HT-22 cells were treated with control or $LaCl_3$ or NAC at the indicated concentration 3 h before the treatment of control or TNF-α/z-VAD for 20 h; cell viability was determined by measuring ATP levels. Data are represented as mean ± standard deviation of duplicates. $^{*}P < 0.01$, $^{**}P < 0.001$ versus control-T + Z. All experiments were repeated three times with similar results.

Authors' Contribution

Shan Liu and Xing Wang contributed equally to this work.

Acknowledgments

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Research Article

Resveratrol Ameliorates Motor Neuron Degeneration and Improves Survival in SOD1^{G93A} Mouse Model of Amyotrophic Lateral Sclerosis

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Resveratrol has recently been used as a supplemental treatment for several neurological and nonneurological diseases. It is not known whether resveratrol has neuroprotective effect on amyotrophic lateral sclerosis (ALS). To assess the effect of resveratrol on the disease, we tested this agent on an ALS model of SOD1^{G93A} transgenic mouse. Rotarod measurement was performed to measure the motor function of the ALS mice. Nissl staining and SMI-32 immunofluorescent staining were used to determine motor neurons survival in the spinal cord of the ALS mice. Hematoxylin-eosin (H&E), succinic dehydrogenase (SDH), and cytochrome oxidase (COX) staining were applied to pathologically analyze the skeletal muscles of the ALS mice. We found that resveratrol treatment significantly delayed the disease onset and prolonged the lifespan of the ALS mice. Furthermore, resveratrol treatment attenuated motor neuron loss, relieved muscle atrophy, and improved mitochondrial function of muscle fibers in the ALS mice. In addition, we demonstrated that resveratrol exerted these neuroprotective effects mainly through increasing the expression of Sirt1, consequently suppressing oxidative stress and downregulating p53 and its related apoptotic pathway. Collectively, our findings suggest that resveratrol might provide a promising therapeutic intervention for ALS.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive and fatal neurodegenerative disease, characterized by selective degeneration and death of central and peripheral motor neurons [1]. Mutations in copper/zinc superoxide dismutase (SOD1) consist of 20% cases of familial ALS (fALS), but the underlying pathogenetic mechanisms in this form of ALS are still largely unknown [2]. Our previous studies have shown a significant elevated homocysteine level in the spinal cord and serum of SOD1^{G93A} mouse model of ALS [3], and application of high dose folic acid may have therapeutic potential for the treatment of the disease [4]. We have also found that autophagy activity is increased in the spinal cord of ALS mice, pointing towards a possible role of autophagy in the pathogenesis of ALS [5]. Currently, there is no effective treatment available for ALS. Therefore, development of novel therapeutic drugs for this devastating disease is in great need.

Resveratrol (3,5,4′-trihydroxy-trans-stilbene) has received considerable attention recently for its potential neuroprotective effects in several neurodegenerative disorders [6]. But there is limited report about the application of resveratrol in ALS. Resveratrol is a polyphenolic compound present in a large number of plant species.

The direct molecular targets of resveratrol *in vitro* and *in vivo* are unknown, but it has been suggested to modulate cellular processes by regulating the expression or posttranscriptional modification of Sirtuin1 (Sirt1) and its substrates peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) and p53 [7–11]. Sirt1 is a NAD-dependent class III

histone deacetylase, which resides mainly in the cell nucleus and targets several histone and nonhistone transcriptional regulators, regulating energy metabolism, cell apoptosis, protein homeostasis, and inflammation [6]. PGC-1α is a potent stimulator of mitochondrial biogenesis and respiration. In recent years, the role of PGC-1α in reactive oxygen species (ROS) metabolism and neurodegenerative diseases has been attracting great attention [12]. The tumor suppressor p53 is activated mainly through posttranslation modifications, including phosphorylation and acetylation. Lysine 382 of p53 (K382-p53) is a well-characterized target for Sirt1 deacetylase activity [6, 12]. Accordingly, we speculate that the activation of Sirt1 by resveratrol might provide neuroprotection in ALS through the suppression of the p53 acetylation and its downstream pathway.

In the current study, we tested the neuroprotective effects of resveratrol in the SOD1^{G93A} mouse of ALS model and determined the activation of Sirt1 and its substrates as a potential underlying mechanism in ALS.

2. Materials and Methods

2.1. Animals. All the animal experiments were carried out in accordance with the guidelines laid down by the NIH in the USA regarding the care and use of animals for experimental procedures and met with ethical standards approved by the Animal Committee of Shanghai Jiaotong University School of Medicine. All efforts were made to reduce the number of animals used and to minimize animal suffering. We used SOD1^{G93A} transgenic mice, a classic animal model of ALS, in our study. The SOD1^{G93A} transgenic mice were obtained from Jackson's laboratory (number 002726). These mice usually became paralyzed in one or more limbs at approximately 90 days of age and were moribund about 30 days later [13]. The SOD1^{G93A} mice were maintained as hemizygotes by crossing transgenic male with wild-type (WT) females of the same B6SJL genetic background.

2.2. Intervention. To assess the effects of resveratrol on the disease onset and survival of ALS mouse model, resveratrol (Sigma; USA) was dissolved into ethanol at 50 mg/mL and further diluted with PBS at 1 : 4 (v/v) before intraperitoneal injection. Twenty male SOD1^{G93A} transgenic mice were randomly divided into two groups: (1) the resveratrol-treated group was intraperitoneally injected with resveratrol at the dose of 25 mg/kg body weight/day ($n = 10$) and (2) the vehicle-treated group, used as control, was intraperitoneally injected with the same dose of vehicle ($n = 10$). The injection was given once a day starting from the 42 days after birth until the day of the animals' death.

To explore the mechanisms of neuroprotection provided by resveratrol, 10 male SOD1^{G93A} mice and 10 age-matched WT littermates were randomly divided into four groups: resveratrol treatment at the dose of 25 mg/kg body weight/day in SOD1^{G93A} transgenic mice (Tg-RSV mice, $n = 5$) or in WT littermates (WT-RSV mice, $n = 5$) and vehicle treatment in SOD1^{G93A} transgenic mice (Tg-Veh mice, $n = 5$) or in WT littermates (WT-Veh mice, $n = 5$). They were intraperitoneally injected once a day from 42 days after birth and were sacrificed at the age of 120 days, when we collected the blood, muscle, and spinal cord tissues for biochemical and histological assays.

2.3. Behavioral Tests

2.3.1. Assessment of Disease Onset. We started to test the rotarod performance when the mice were at the age of 70 days. The rotarod performance was examined every other day to determine the time period that the mice remained on the rotating rod (4 cm diameter, 20 rpm). When the mouse could not stay on the rotarod for 5 minutes, it was defined as the date of disease onset [14].

2.3.2. Assessment of Lifespan. The mice were sacrificed, if they could not right themselves within 30 seconds being placed on a flat surface and the data was recorded as the age of death [15].

2.4. Pathological Analysis of Skeletal Muscles. Biopsied gastrocnemius muscles ($5 \times 5 \times 10$ mm^3) were dissected out from the right leg of an anaesthetized animal and immersed immediately in isopentane cooled in liquid nitrogen. Serial cryostat sections were cut at 10 μm and stained by hematoxylin and eosin (H&E). In addition, mitochondrial function was demonstrated by succinic dehydrogenase (SDH) staining and cytochrome oxidase (COX) staining [16].

2.5. Biochemical Analysis of Blood Samples

2.5.1. Preparation of Blood Samples. Under deep anesthesia, blood (200 μL per mouse) was collected from the eyeballs of mice at the age of 120 days. The blood was centrifuged at 4°C for 10 min at 3,000 rpm to separate the serum samples.

2.5.2. Lipid Peroxidation Assay. For lipid peroxidation assay, we used a commercial kit (S0131; Beyotime; China) to measure the concentration of malondialdehyde (MDA) in spinal cord tissues and serum samples according to the manual instruction provided by the company.

2.6. Histopathological Analysis of Motor Neurons in Lumbar Spinal Cord. The mice were anesthetized and perfused transcardially with phosphate buffered saline (PBS) at the age of 120 days. For histopathological analysis, the spinal cord (L4-5) was removed, postfixed, and dehydrated in 15% and 30% sucrose each for 24 hours. For western blot assay, the spinal cord (C1-L3) was quickly removed and preserved in liquid nitrogen for further analysis.

The lumbar spinal cords (L4-5) were transversely sectioned at 10 μm with a Leica cryostat. A total of 250 serial sections in each mouse were cut on gelatin coated slides and frozen at −80°C until use.

For immunofluorescence assay, transverse cryosections were blocked and incubated overnight at 4°C with a mouse monoclonal antibody against nonphosphorylated neurofilament H (SMI-32; 1 : 1500; Covance; USA). Then, sections

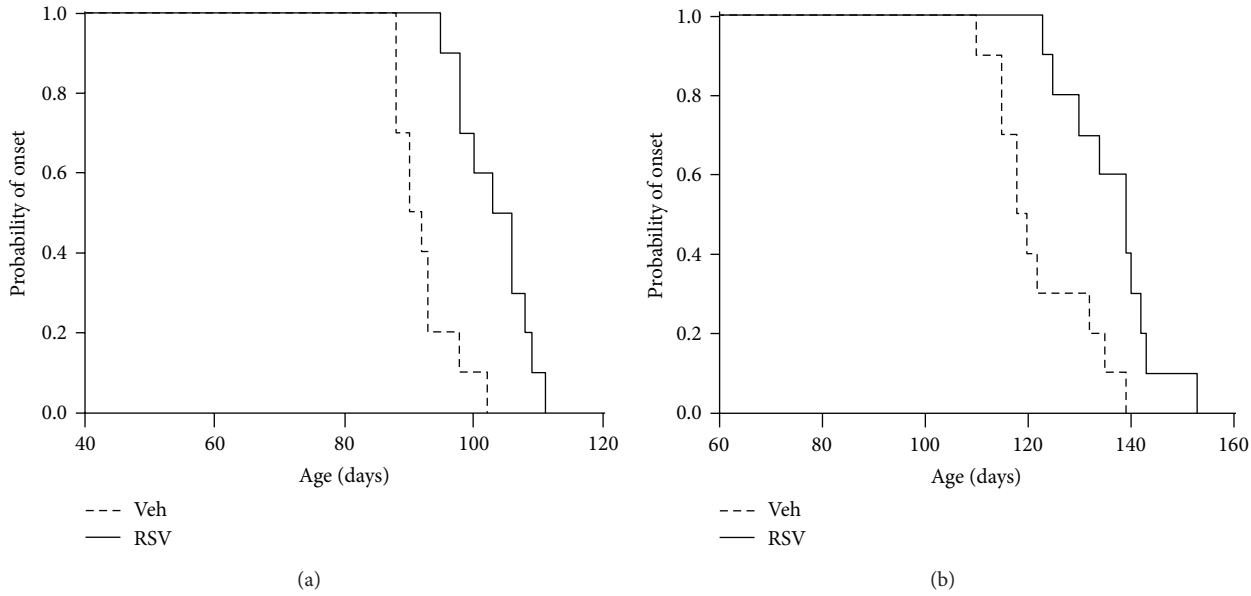

Figure 1: The benefit effects of resveratrol on disease onset and lifespan in the SOD1^{G93A} mice. Kaplan-Meier survival curves indicated, respectively, the probability of (a) disease onset and (b) survival in the vehicle or resveratrol-treated ALS mice. $N = 10$ in each group.

were thoroughly washed and incubated for 2 hours at room temperature with the fluorescent-conjugated antibody. Fluorescence images were taken by an inverted fluorescent microscope (Olympus IX81; Japan).

For Nissl staining, every 5th section was selected from the serial sections to have 50 sections in each mouse to be stained with 1% toluidine blue at 55°C for 2 minutes. Both sides of motor neurons located in the anterior horn, which had a maximum diameter no less than 20 μm and contained a distinct nucleus, were counted by investigators in a blinded manner [17].

2.7. Western Blotting. Fresh spinal cord tissues were dissolved and homogenized in RIPA lysis buffer containing 1% protease inhibitor phenylmethanesulfonyl fluoride (PMSF) (Sangon Biotech; China). Proteins were loaded and separated in SDS-PAGE gel and then transferred to polyvinylidene fluoride membranes. After being blocked, membranes were incubated at 4°C overnight with the following antibodies: anti-Sirt1 (1 : 1000; Santa Cruz; USA), anti-PGC1-α (1 : 200; Santa Cruz; USA), anti-p53 (1 : 1000; Cell Signaling; USA), anti-acetyl-p53 (1 : 1000; Millipore; USA), anti-Bax (1 : 1000; Cell Signaling; USA), anti-Bcl-2 (1 : 1000; Cell Signaling; USA), anti-caspase-3 (1 : 1000; Cell Signaling; USA), anti-Cytochrome C (Cyt C) (1 : 4000; Abcam; USA), and anti-PARP (1 : 1000; Santa Cruz; USA). After being washed thoroughly, the membranes were incubated with the appropriate secondary antibody for 2 hours. Protein bands were visualized by ECL (Pierce; USA) and an image analyzer (Quantity One-4.2.0; Bio-Rad; USA) was used to quantify the density of interested bands.

2.8. Statistics. SPSS 17.0 was used for statistical calculations. Disease onset and survival statistics were performed by Kaplan-Meier survival curves and the data were analyzed using the log-rank test, generating a χ^2 value to test for significance. Other data in different groups of animals were assessed by a one-way analysis of variance (ANOVA). Results were expressed as means ± SEM values. P values less than 0.05 were considered significant.

3. Results

3.1. Resveratrol Delayed Disease Onset and Extended the Lifespan in the SOD1^{G93A} Mice. SOD1^{G93A} transgenic mice treated with resveratrol showed significant delay of disease onset compared with vehicle-treated transgenic mice (103.40 ± 5.42 versus 92.20 ± 4.64 days, $\chi^2 = 11.16$, $P < 0.01$) (Figure 1(a)). The lifespan of SOD1^{G93A} mice treated with resveratrol was 14.40 days longer than the vehicle-treated counterparts (136.80 ± 9.02 versus 122.40 ± 9.63 days, $\chi^2 = 8.29$, $P < 0.01$) (Figure 1(b)).

3.2. Resveratrol Ameliorated Motor Neuron Loss in the SOD1^{G93A} Mice. From the immunofluorescence images, motor neurons in the anterior horn of lumbar spinal cord in Tg-Veh mice were fewer and smaller than those of the WT-Veh mice, while resveratrol treatment significantly protected the motor neurons in the Tg-RSV mice (Figure 2(a)). We then quantitatively counted in a blind manner the number of Nissl's stained-motor neurons. Compared with the WT-Veh littermates, only 33.19% of the motor neurons remained at the same level of the spinal cord in the Tg-Veh mice (264.25 ± 21.25 versus 796.25 ± 42.61, $P < 0.001$). However, resveratrol injection in the Tg-RSV mice retained 81.48% of the motor neurons compared with the Tg-Veh mice (438.75 ± 41.31 versus 796.25 ± 42.61, $P < 0.01$). No significant difference

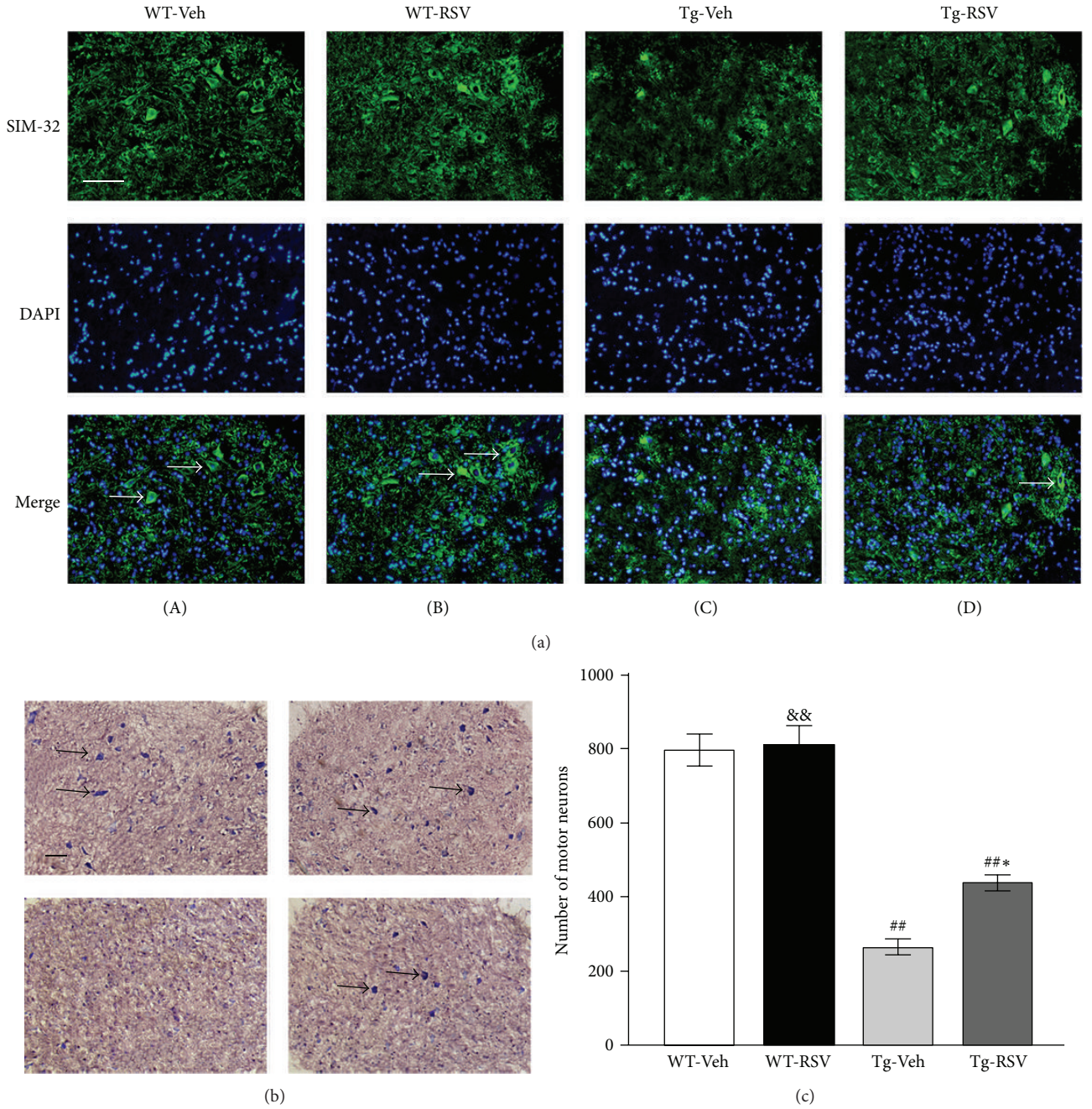

Figure 2: Resveratrol administration prevented motor neuron death in SOD1^{G93A} mice at the age of 120 days. (a) Immunofluorescent staining of motor neurons (white arrows) with SMI-32 (green) antibody in the anterior horn of spinal cord from different groups of mice. (b) Photomicrographs of Nissl's stained-motor neurons (black arrows) in the anterior horn of the spinal cord of SOD1^{G93A} mice in different groups. (A) WT-Veh group, (B) WT-RSV group, (C) Tg-Veh group, and (D) Tg-RSV group. (c) Quantification of motor neurons in the anterior horn of lumbar spinal cord in different groups. The values are means ± SEM. $^{\#\#}P < 0.001$ versus WT-Veh group; $^{*}P < 0.01$ versus Tg-Veh group; $^{\&\&}P < 0.001$ versus Tg-RSV group. $N = 5$ in each group. Scale bar = 100 μm.

was found between the WT-RSV and WT-Veh mice (811.75 ± 50.20 versus 796.25 ± 42.61, $P > 0.05$) (Figures 2(b) and 2(c)).

3.3. Resveratrol Ameliorated the Atrophy and Mitochondrial Dysfunction in the Muscle Fibers of SOD1^{G93A} Mice. Using H&E staining, we found that the ALS mice treated with vehicle showed significant reduction in cross-section area of gastrocnemius muscle fibers compared with their age-matched WT-Veh littermates. Resveratrol administration increased the cross-section area gastrocnemius muscle fibers in the Tg-RSV mice compared with the Tg-Veh counterparts

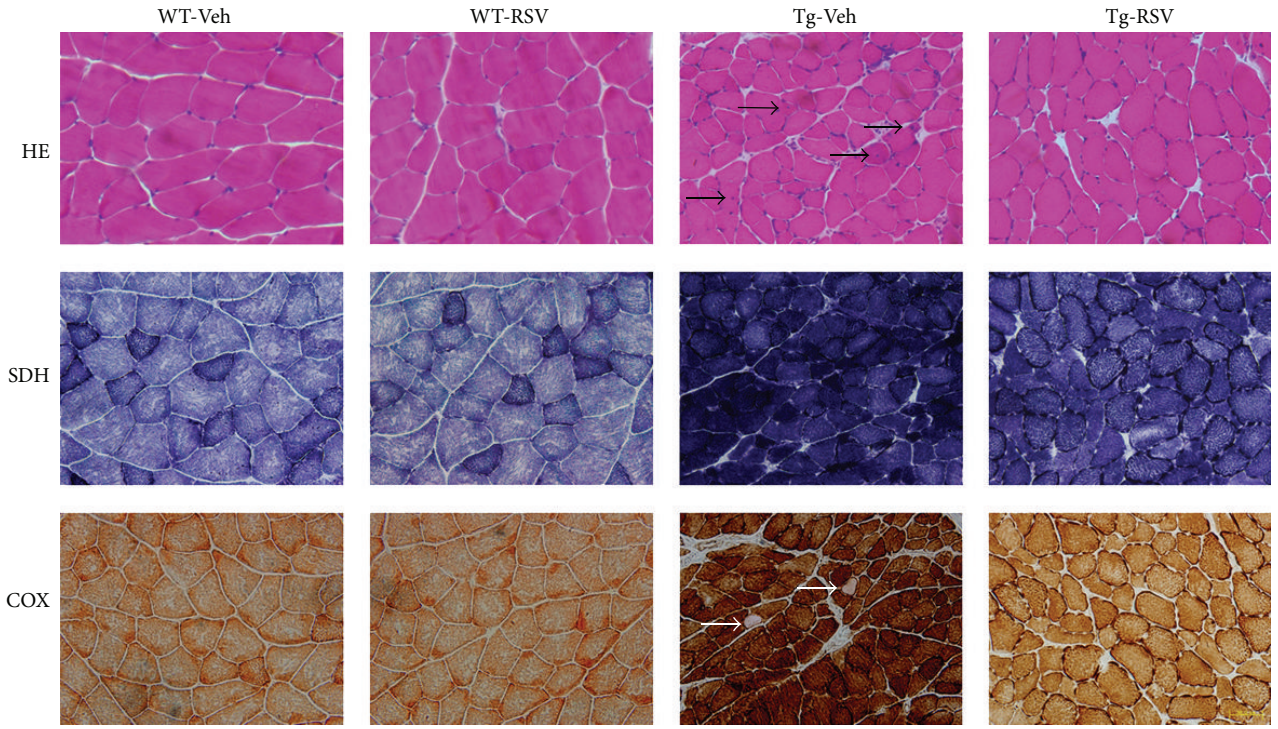

Figure 3: Effects of resveratrol on the gastrocnemius muscles from different groups of mice at the age of 120 days. Black arrows indicated the myofibers with internal myonuclei; white arrows indicated myofibers with absent COX staining.

(Figure 3). We did not find central nuclei in the Tg-RSV mice (Figure 3).

SDH staining is regarded as the standard screening reaction for mitochondria [14]. Notably, the SDH staining of the metabolic activity of skeletal muscles showed a high level of oxidative stress in the Tg-Veh mice as compared with the WT-Veh littermates (Figure 3). However, the increase of SDH staining was significantly attenuated in the resveratrol-injected Tg-RSV mice.

COX is another mitochondrial enzyme, which reflects the capacity of cells to undergo mitochondrial electron transport and oxidative phosphorylation [18]. We observed that, in the Tg-Veh mice, some muscle fibers had no staining for COX. The deficiency of COX may indicate the overproduction of ROS, which renders the mitochondrial genome susceptible to ROS-induced injury. However, in the Tg-RSV mice, it seemed that most of the muscle fibers were COX positive.

3.4. Resveratrol Regulated the Expression of Sirt1 and PGC1-α and Improved the Lipid Peroxidation in the SOD1^{G93A} Mice. The level of Sirt1 in the Tg-Veh mice was nearly half as seen in the WT-Veh mice (0.98 ± 0.09 versus 1.91 ± 0.15, $P < 0.01$). After the chronic treatment with resveratrol, the Sirt1 levels in the spinal cords of Tg-RSV and WT-RSV mice were increased by 49.22% and 47.96%, respectively (1.46 ± 0.09 versus 0.98 ± 0.09, $P < 0.05$ and 2.85 ± 0.19 versus 1.91 ± 0.15, $P < 0.01$) (Figures 4(a) and 4(c)). Further, we found that PGC-1α level in the spinal cords of Tg-Veh mice was increased up to 276.42% compared with the WT-Veh littermates, whereas resveratrol administration significantly suppressed the expression of PGC-1α, making it near the level of WT-Veh littermates. There was no significant difference of PGC-1α between WT-Veh and WT-RSV mice (Figures 4(b) and 4(d)).

In the Tg-Veh mice, the level of MDA was nearly 4-fold that in the WT-Veh mice (20.30 ± 2.16 versus 5.88 ± 1.32 μmol/L, $P < 0.001$), whereas resveratrol treatment significantly decreased the MDA level by 33.70% in the ALS mice (13.46 ± 1.29 versus 20.30 ± 2.16, $P < 0.01$). Similarly, in the spinal cords of Tg-RSV mice, the MDA level was moderately lower than that in the Tg-Veh mice, although the statistical analysis showed no significant difference.

3.5. Resveratrol Inhibited p53 and Its Downstream Apoptotic Pathway in the ALS Mice. The quantitative analysis showed that the level of p53 in the Tg-Veh mice was nearly 4-fold that in the WT-Veh mice. The level of p53 was decreased by 58.80% after the resveratrol treatment in the Tg-RSV mice (1.71 ± 0.24 versus 4.15 ± 0.27, $P < 0.001$). Similarly, the level of acetyl-p53 in the Tg-Veh mice was 74.04% higher than that of the WT-Veh mice (1.81 ± 0.07 versus 1.04 ± 0.14, $P < 0.01$) but was decreased by 20.99% after the resveratrol treatment (1.43 ± 0.09 versus 1.81 ± 0.07, $P < 0.05$).

We then determined the mitochondria-dependent apoptosis protein levels of Bax, Bcl-2, cleaved caspase-3, Cyt C, and cleaved PARP. The Western blot results showed that the antiapoptotic protein level of Bcl-2 was considerably reduced in the Tg-Veh mice compared with the WT-Veh mice, while resveratrol treatment in the Tg-RSV mice increased the level of Bcl-2. Conversely, Bax, cleaved caspase-3, Cyt C, and

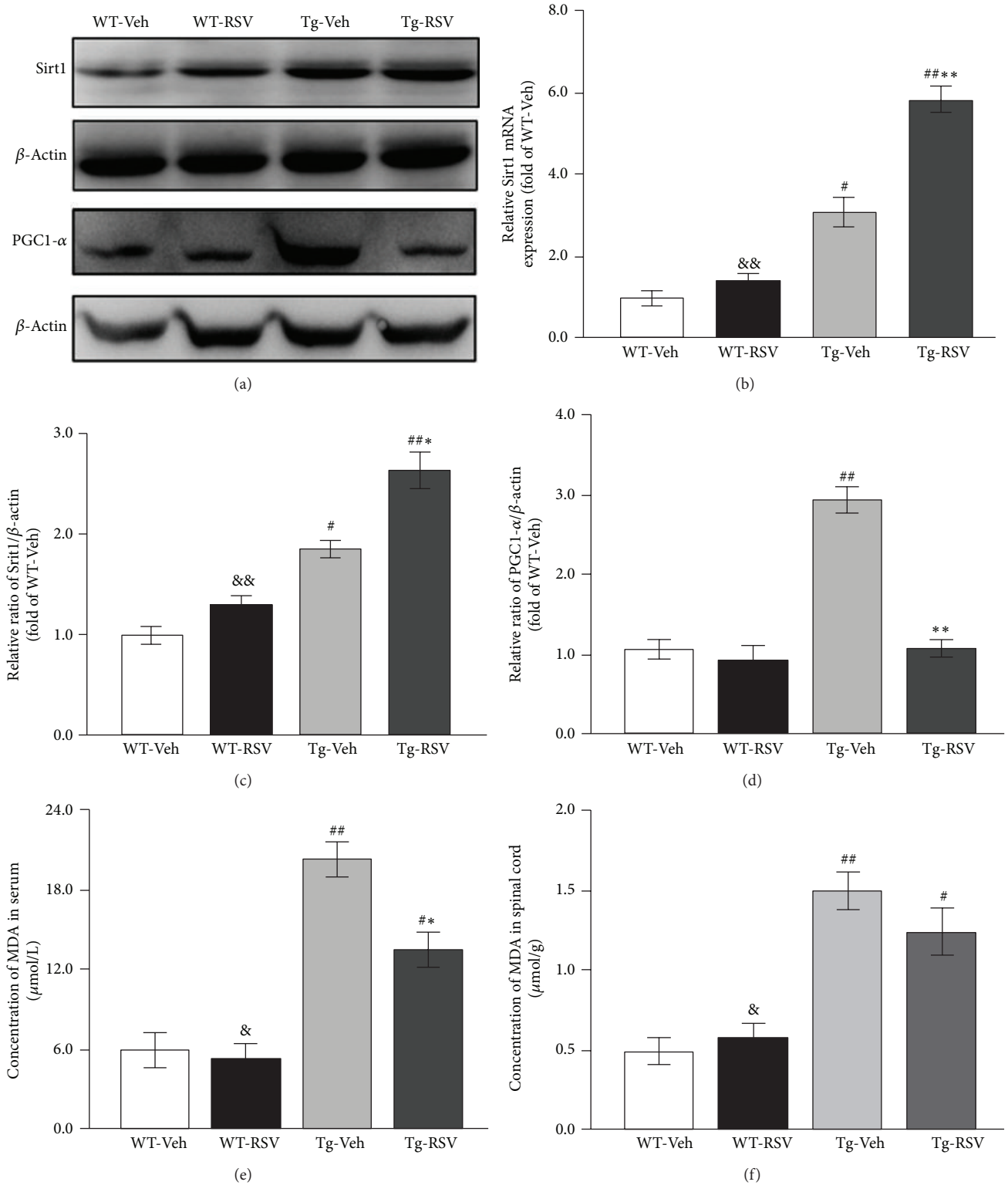

FIGURE 4: Effects of resveratrol on oxidative capacity in different groups of mice. (a) Western blot assay of Sirt1 and PGC1-α in the spinal cord tissues of WT and transgenic mice treated or untreated with resveratrol. (b) Relative Sirt1 mRNA expression in the spinal cords of different groups of mice. (c-d) Quantitative analysis of the protein expression of (c) SIRT1 and (d) PGC1-α in the spinal cords. (e-f) MDA concentration in (e) serum and (f) spinal cords from different groups of mice. The values are means ± SEM. $^{\#}P < 0.01$ and $^{\#\#}P < 0.001$ versus WT-Veh group; $^{*}P < 0.01$ and $^{**}P < 0.001$ versus Tg-Veh group; $^{\&}P < 0.01$ and $^{\&\&}P < 0.001$ versus Tg-RSV group. $N = 5$ in each group.

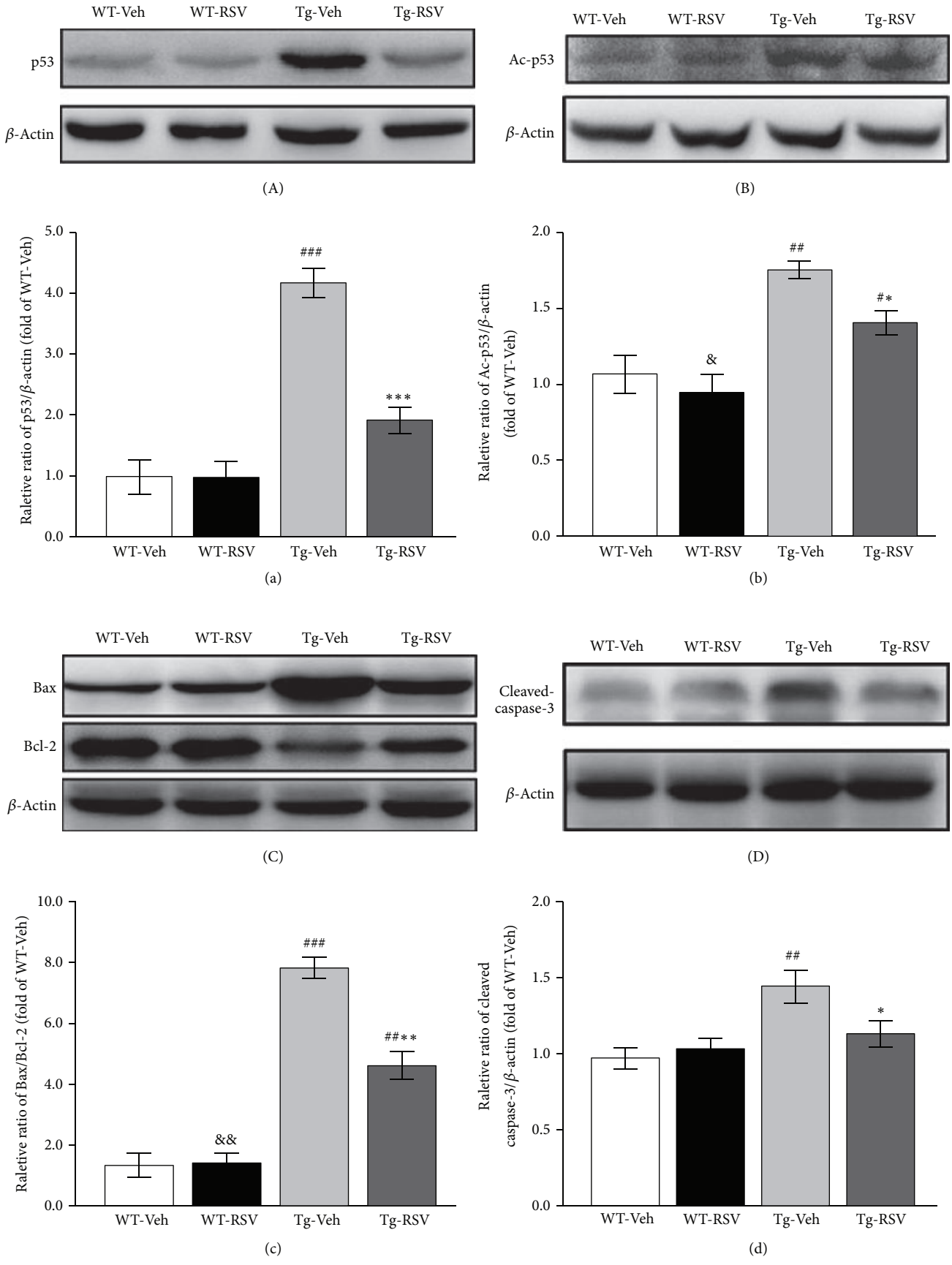

Figure 5: Continued.

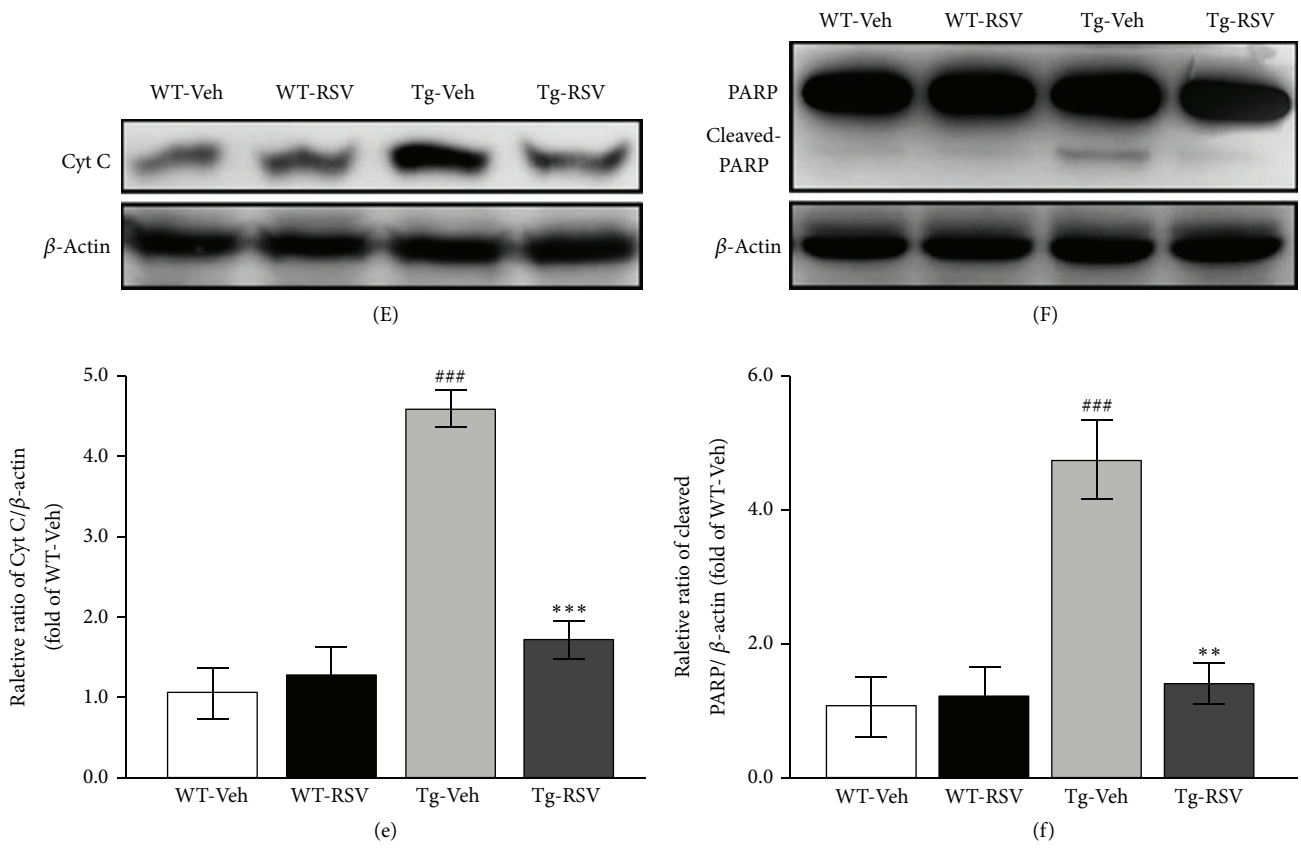

Figure 5: Expression of apoptosis-related molecules in WT and transgenic mice treated with vehicle or resveratrol. (A–F) Western blot assay of (A) p53, (B) acetyl-p53, (C) Bax/Bcl-2, (D) cleaved caspase-3, (E) Cyt C, and (F) PARP from the spinal cord tissues in different groups of mice. (a–f) Quantitative data of the expression of (a) p53, (b) acetyl-p53, (c) Bax/Bcl-2, (d) cleaved caspase-3, (e) Cyt C, and (f) PARP in the four groups of mice. The values are means ± SEM. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ versus WT-Veh group; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus Tg-Veh group; $^{\&}P < 0.05$ and $^{\&\&}P < 0.01$ versus Tg-RSV group. $N = 5$ in each group.

cleaved PARP were markedly elevated in the Tg-Veh mice, and resveratrol administration could suppress the expression of these proteins in the Tg-RSV mice. However, all of the apoptosis-related molecules were not affected in the WT-RSV mice compared with the WT-Veh mice (Figure 5(B)).

4. Discussion

Our study provides first evidence that resveratrol treatment can delay the onset of disease and prolong the lifespan in a well-established ALS mouse model, probably through the protection of motor neurons and skeletal muscles. The neuroprotective effects of resveratrol may be related to its biological role to increase the expression of Sirt1, suppress the expression of PGC-1α, and inhibit oxidative stress in the spinal cord of the ALS mice. Furthermore, resveratrol has significant antiapoptotic effects by upregulating Bcl-2 and inhibiting p53 and its downstream apoptotic pathway.

Resveratrol has been one of the most extensively studied polyphenols recently owing to its potent therapeutic activities. However, the *in vivo* biological effects of resveratrol appear strongly limited by its low bioavailability [19]. In the present study, we put our efforts to improve its bioavailability. Firstly, reseratrol was kept away from light, since it is an extremely photosensitive compound. In addition, we used 10% ethanol in PBS as vehicle to enhance the solubility of resveratrol. Moreover, we gave the solution through interperitioneal injection, reducing the first pass effect.

Oxidative stress and mitochondrial dysfunction have been regarded as important risk factors for pathogenesis of ALS [20, 21]. It was reported that resveratrol could improve mitochondrial function and protect against metabolic disease by activating Sirt1 and PGC-1α in C57B1/6J mice [22]. In this study, we found that resveratrol also activated Sirt1 and improved mitochondrial function in the ALS mice. MDA, which reflects the severity of oxidative stress, was significantly decreased in the sera and in the spinal cords of Tg-RSV mice compared with the Tg-Veh mice. We considered that the antioxidant effect of resveratrol resulted partly, at least, from the activation of Sirt1.

Previous works reported that the expression of PGC-1α was regulated by Sirt1 in different tissues [23, 24]. Overexpression of PGC-1α could significantly improve motor function and prolong the lifespan of ALS mice, which indicate that PGC-1α may play a protective role in ALS [25]. Moreover, it

has been suggested that AMPK, p38 MARK, and ROS were involved in the regulation of PGC-1α [11, 26]. PGC-1α can be induced by elevated ROS as a responsive protective effort in neural cells [27]. In our study, we found that the level of PGC-1α was dramatically increased in Tg-Veh mice compared with WT-Veh or WT-RSV mice, indicating that the elevated ROS might be involved in the increase of PGC-1α in the ALS mice (Figure 4(a)). We also demonstrated that the level of ROS was suppressed after the resveratrol treatment (Figures 4(e) and 4(f)), and then the level of PGC-1α was significantly reduced (Figure 4(a)) which we believe that it might be the result of the decreased ROS level in Tg-RSV mice.

Increasing evidences support that apoptosis activation is the terminal process of motor neuron death in ALS [28]. p53 can bind to Bcl-2 and thus promote opening of the mitochondrial pore with mitochondrial permeabilization, release of cytochrome *c*, and activation of caspase-3 and leads to apoptotic cell death [1]. p53 is a sequence-specific transcription factor that is dramatically increased in response to a variety of cellular stresses, and its activation may cause cell death by directly inducing mitochondrial permeability and apoptosis [3, 29]. Now, Sirt1 is believed to regulate apoptotic thresholds by deacetylating p53 [6, 12]. In our study, we documented that resveratrol can have neuroprotective effect through the Sirtl mediated anti-p53 apoptotic pathway in the ALS mice.

Aberrant protein misfolding is known to contribute to the pathogenesis of ALS and possibly is related to the defect of autophagy-lysosomal pathway, which may exacerbate the pathological processing through apoptotic and other mechanisms in the disease [30]. We confirm in our study that the strong inhibition effects of resveratrol on apoptosis through the upregulation of Bcl-2 and suppression of Bax, cleaved caspase-3, p53, and acetyl-p53 as well as its down-stream mitochondria-dependent apoptotic pathway. We believe that these collective effects prevent the motor neurons from degeneration in ALS.

5. Conclusions

Taken together, our findings indicate that resveratrol could delay the onset of disease and prolong the lifespan of $SOD1^{G93A}$ mice. Resveratrol can significantly attenuate the motor neuron loss and reduce the muscle atrophy and dysfunction in the ALS mice. It is likely that the antioxidant and antiapoptotic effects of resveratrol are the major beneficial roles of this compound to play against ALS.

Abbreviations

ALS: Amyotrophic lateral sclerosis
COX: Cytochrome oxidase
Cyt C: Cytochrome C
H&E: Hematoxylin and eosin
MD: Malondialdehyde
PBS: Phosphate buffered saline
PGC1-α: Peroxisome proliferator-activated receptor-γ coactivator-1α
PMSF: Phenylmethanesulfonyl fluoride
ROS: Reactive oxygen species
SDH: Succinic dehydrogenase
Sirt1: Sirtuin1
SMI-32: Nonphosphorylated neurofilament
SOD1: Copper/zinc superoxide dismutase
WT: Wild-type.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

The Role of microRNAs in the Regulation of Apoptosis in Lung Cancer and Its Application in Cancer Treatment

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Lung cancer remains to be one of the most common and serious types of cancer worldwide. While treatment is available, the survival rate of this cancer is still critically low due to late stage diagnosis and high frequency of drug resistance, thus highlighting the pressing need for a greater understanding of the molecular mechanisms involved in lung carcinogenesis. Studies in the past years have evidenced that microRNAs (miRNAs) are critical players in the regulation of various biological functions, including apoptosis, which is a process frequently evaded in cancer progression. Recently, miRNAs were demonstrated to possess proapoptotic or antiapoptotic abilities through the targeting of oncogenes or tumor suppressor genes. This review examines the involvement of miRNAs in the apoptotic process of lung cancer and will also touch on the promising evidence supporting the role of miRNAs in regulating sensitivity to anticancer treatment.

1. Introduction

Lung cancer remains a major health problem worldwide. In 2012 lung cancer was the most commonly diagnosed cancer worldwide making up 13.0% of the total incidence of cancer. It was also the most common cause of death from cancer worldwide, accounting for nearly one in five cancer deaths (19.4% of the total) [1]. Lung cancer is clinically divided into two main groups based upon the size and appearance of malignant cells: small cell lung cancer (SCLC) (16.8%) and non-small cell lung cancer (NSCLC) (80.4%) [2]. The most effective option for treatment of lung cancer is surgical resection, when feasible [3]. However, majority of patients are diagnosed at an advanced or metastatic stage of disease in which case chemotherapy and/or concurrent administration of chemotherapy and radiation is the most beneficial form of treatment [4]. Nevertheless, even with treatment, the 5-year survival rate in patients is only 16.6% [5], with poor survival rates mainly being attributed to late stage diagnosis and high frequency of drug resistance. Obtaining a better understanding regarding the molecular mechanisms involved in lung carcinogenesis is of utmost importance in the aim to identify the diagnostic and prognostic markers for early detection and targeted treatment of lung cancer.

Apoptosis plays an important role during development and in the maintenance of multicellular organisms through the removal of damaged, aged, or autoimmune cells [6]. The apoptotic process can be divided into the extrinsic and intrinsic pathway. Each pathway will ultimately result in the activation of cell death proteases, which in turn initiates a cascade of proteolysis involving effector caspases that carries out the completion of the apoptotic process [7]. In contrast to normal cells, cancer cells have the ability to evade apoptosis to promote cell survival under the conditions of environmental stress. There are a number of mechanisms by which cancer cells are able to suppress apoptosis. For example, the tumor suppressor gene *p53* is a widely mutated gene in human tumorigenesis [8]. *p53* mutation will inhibit the activation of DNA repair proteins leading to a decrease in the initiation of apoptosis [7], allowing for cells to divide

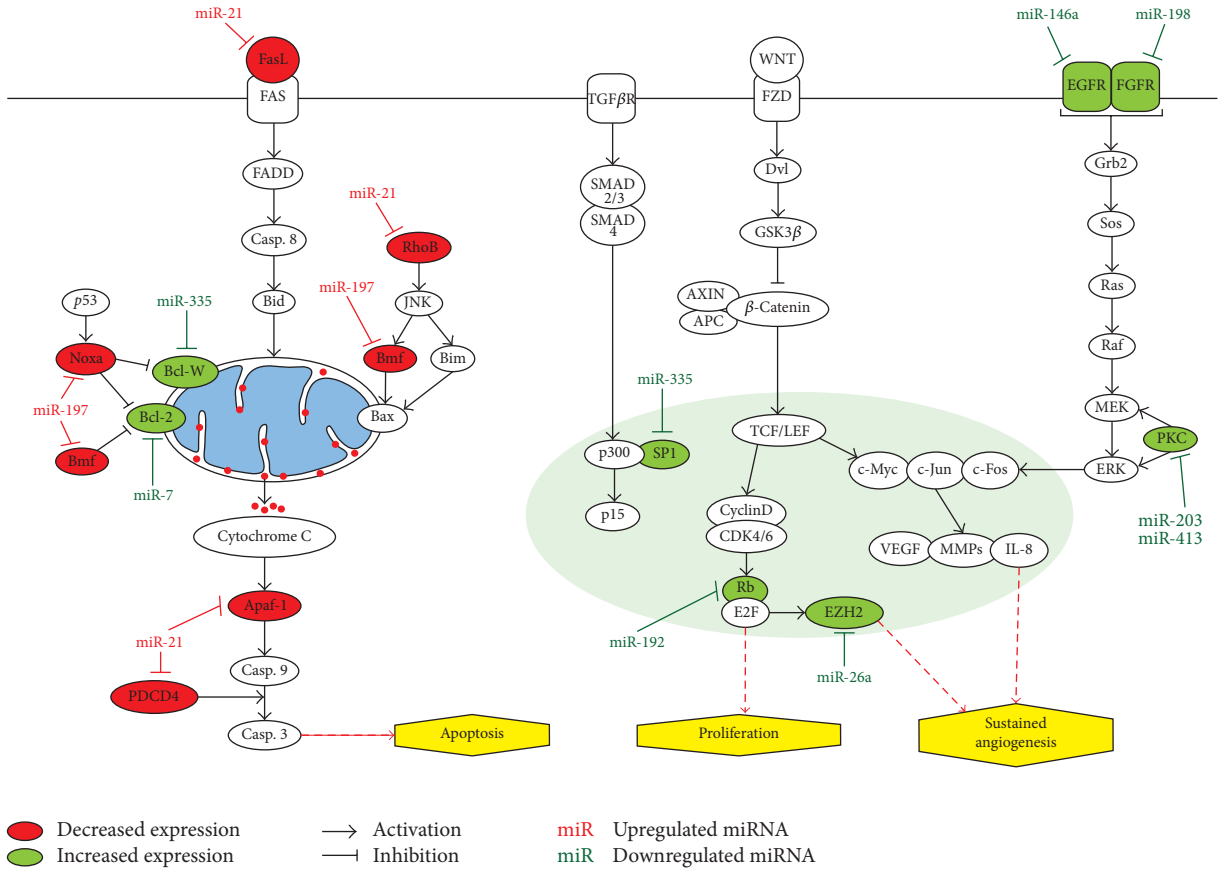

Figure 1: Scheme depicting up- and downregulated miRNAs and the roles they play in various biological pathways including apoptosis, proliferation, and angiogenesis.

and grow uncontrollably, forming malignant tumors. Furthermore, cancer cells are able to disrupt the balance between pro- (*BCL-2*, *BCL-XL*) and antiapoptotic factors (*BAX*, *BIM*, and *PUMA*) [9]. Increased expression of proapoptotic Bcl-2 protein contributes not only to the development of cancer but also to resistance against a wide variety of anticancer agents, such as cisplatin (DDP) and paclitaxel [10–12].

MicroRNAs (miRNAs) are a subset of noncoding RNAs of about 20 to 25 nucleotides long which posttranscriptionally regulate gene expression via inhibition of mRNA translation, by binding to specific target sites in their 3′-untranslated region (3′UTR), or inducing degradation of target mRNA through cleavage [13]. An individual miRNA is able to modulate the expression of multiple genes; correspondingly, a single target can be modulated by many miRNAs [14]. MiRNAs were reported to be involved in a vast range of biological processes, including apoptosis (see Figure 1) [15–22]. As miRNAs play a key role in an assortment of biological processes, an altered miRNA expression is likely to contribute to human diseases including cancer [23]. Previous studies have shown that compared to normal tissues, malignant tumors and tumor cell lines were found to have widespread deregulated miRNA expression [24–28]. MiRNAs are critical apoptosis regulators in tumorigenesis and cancer cells are able to manipulate miRNAs to regulate cell survival in oncogenesis. Many studies carried out in the past several years are aimed at elucidating the specific miRNAs associated with apoptosis in cancer and their related target genes. In this review we will examine the recent progress of research on miRNA-mediated regulation of apoptosis in lung cancer and its future therapeutic applications.

2. Antiapoptotic miRNAs

Evasion of apoptosis is a significant hallmark of tumor progression, and one mechanism by which miRNAs influence development of cancer is through the regulation of the apoptotic process as shown in various studies [29–32]. miRNA expression can be either upregulated or downregulated and evidence has shown that dysregulated miRNAs can behave as oncogenes or tumor suppressor genes in lung cancers [18, 28, 33]. Amplification of miRNAs can lead to the downregulation of tumor suppressors or other genes that are involved in apoptosis [34].

miR-197. For example, the expression of miR-197 is increased in cancer tissues in comparison to normal specimens. Fiori et al. (2014) demonstrated that knockdown of miR-197 in

NIH-H460 and A549 cells promoted induction of apoptosis, evident by the observation of caspases 3–7 activation and increased apoptotic population by Annexin staining. Furthermore, the direct interaction of miR-197 with the 3′UTR of *BMF* and *NOXA* was demonstrated by the luciferase reporter assay [35]. When activated by intra- or extracellular stimuli, proapoptotic Bmf binds to and neutralizes antiapoptotic Bcl-2 family members on the mitochondrial membrane, thus allowing proapoptotic proteins Bak and Bax to dimerize and promote the release of cytochrome c, ultimately leading to cell death [36]. Noxa is a BH-3 only proapoptotic protein transcriptionally activated by *p53*. Collectively, miR-197 is able to act upon different levels of the *p53* pathway to counteract the induction of apoptosis, thus allowing cells to proliferate uncontrollably [35].

miR-21. miR-21 is found to be frequently upregulated in a number of cancers; however its potential role in tumorigenesis *in vivo* is not fully explored. Using transgenic mice with loss-of-function and gain-of-function miR-21 alleles, Hatley and colleagues elucidated the role of miR-21 in NSCLC pathogenesis *in vivo* [37]. It was determined that miR-21 regulates tumor proliferation and survival, which are two integral components of NSCLC pathogenesis, by targeting negative regulators of the RAS pathway as well as by targeting proapoptotic genes [37]. In regards to the apoptotic pathway, overexpression of miR-21 *in vivo* leads to decreased protein levels of Apaf-1, an important component of the intrinsic mitochondrial apoptotic pathway, as well as decreased expression of FasL, a key initiator of the extrinsic apoptotic pathway. Furthermore, *RHOB*, with a tumor suppressor role, is a target of miR-21 and its dysregulation leads to an increase in cell growth and inhibition of apoptosis [38]. Together these results suggest that relieving miR-21 downregulation of these proapoptotic and tumor suppressor genes could provide a means to enhance the effect of current chemotherapy.

miR-212. Acetylcholinesterase (AChE), a component of the cholinergic system, has the ability to influence apoptotic sensitivity both *in vitro* and *in vivo* [39–41]. In NSCLC tissues AChE levels are low and are associated with tumor aggressiveness, increase risk of postoperative recurrence, and low survival rate [42]. Lu et al. (2013) determined that *AChE* expression in NSCLC was posttranscriptionally modulated by miR-212 binding to its 3′UTR. Interestingly, alterations in neither AChE nor miR-212 expression significantly affected cell survival; however it was observed that during DDP-induced apoptosis miR-212 levels were reduced with a concurrent increase in AChE protein levels. This suggests that miR-212 plays a role in DDP resistance by directly inhibiting AChE and preventing apoptosis. Therefore, interference against miR-212 may potentially be a means to improve the pharmacotoxicological profile of DDP in NSCLC [43].

miR-17-5p and miR-20a. The miR-17-92 cluster, which is composed of seven miRNAs and resides in intron 3 of the *C13orf25* gene at 13q31.3, is frequently overexpressed in lung cancers [44]. Matsubara et al. (2007) demonstrated that inhibition of two components of the miR-17-92 cluster, miR-17-5p, and miR-20a, with antisense oligonucleotides can induce apoptosis selectively in lung cancer cells that overexpress miR-17-92 [45]. Previously, miR-17-5p and miR-20a have been shown to directly target *E2F1* [46]; thus inhibition of these miRNAs may cause the induction of apoptosis in part through the induction of *E2F1* and subsequent cell cycle progression into *S* phase [47]. However additional studies would have to be carried out to determine the actual targets for the miR-17-92 cluster to gain a better understanding of the development of this cancer.

3. Proapoptotic miRNAs

MiRNAs that are downregulated are considered tumor suppressor genes. Tumor suppressor miRNAs usually prevent tumor development by negatively regulating oncogenes and/or genes that control cell differentiation or apoptosis [48]. MiRNAs that act as tumor suppressors can be downregulated as a result of deletions, epigenetic silencing, or loss of expression of transcription factors (see Table 2) [49].

3.1. B-Cell Lymphocyte 2 (BCL-2) Family Related miRNAs. Members of the evolutionarily conserved *BCL-2* family are thought to be the central regulators of apoptosis. The expression level of *BCL-2* differs for different cell types; however high levels and aberrant patterns of *BCL-2* expression were reported in a wide variety of human cancers, including lung cancer [50]. Elevation of Bcl-2 protein expression contributes not only to the development of cancer but also to resistance against a wide variety of anticancer agents [10–12].

miR-7. Xiong et al. showed that miR-7 was downregulated in NSCLC cells and *BCL-2* was identified as a direct target [51]. Transfection of miR-7 in A549 cells led to a significant reduction in endogenous *BCL-2* mRNA and protein levels and correspondingly led to increase in the activities of caspase-3 and caspase-7 in cells with apoptotic nuclei [51]. These results thus provide evidence that *BCL-2* may be involved in miR-7 mediated apoptosis induction in A549 cells.

miR-335. BCL-W, another antiapoptotic member of the *BCL-2* family, was found to be a direct target of miR-335 [52]. miR-335 was downregulated in A549 and NCI-H1299 cells, and upregulation of this miRNA via transfection of miR-335 mimics led to a suppression of cell invasiveness and promotion of apoptosis. Furthermore Dyanan and Tjian (1983) discovered that miR-335 directly targeted *SP1* gene, a member of the family of Sp/Kruppel-like transfection factors [53], which can enhance the activity of promoters of numerous genes involved in cell proliferation, apoptosis, differentiation, cell cycle, progression, and oncogenesis thus regulating these genes' expression [54].

miR-608. Studies in our lab identified a *BCL-XL*-induced miRNA, miR-608, involved in the regulation of cell death in A549 and SK-LU-1 cells [55]. *BCL-XL*, a major prototype of the antiapoptotic *BCL-2* gene was found to be overexpressed in NSCLCs [56]. Silencing of *BCL-XL* in A549 and SK-LU-1 led to the significant dysregulation of a number of miRNAs,

as determined through miRNA microarray, with miR-608 being the most upregulated miRNA. Upregulation of miR-608 in A549 and SK-LU-1 via miR-608 mimics led to an increase in apoptotic population, as determined by Annexin-V FITC apoptotic assay, in comparison to NP-69 cells (normal human nasopharyngeal epithelial cell line) (see Table 1) [55]. Bioinformatics analysis determined that miR-608 may be associated with various signaling pathways, primarily the phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT), wingless-type MMTV integration site family (WNT), transforming growth factor (TGF-β), mitogen activated protein kinase (MAPK), and the intrinsic pathway. However the true targets of miR-608 and its direct effects on the apoptotic process is yet to be determined.

3.2. Protein Kinase C (PKC) Family Related miRNAs . PKC is a serine/threonine kinase that is involved in various signal transduction pathways including those related to cellular proliferation, differentiation, and apoptosis [57–59]. PKC plays a role in lung cancer and levels of PKC proteins were found to be increased in various cell lines (A549, NCI-H1355, NCI-H1703, NCI-H157, and NCI-H1155) in comparison to primary normal human bronchial epithelial cells (NHBE) [60].

miR-203. To determine the role that miR-203 can play in the influence of cellular function, putative target prediction was carried out and PKC-α was determined to be a target [61]. Luciferase reporter assay further revealed miRNA-203 direct binding of the 3′UTR of *PKC-α* mRNA transcript. miR-203 negatively regulated proliferation and migration through the repression of *PKC-α*, and miR-203 was also able to modulate cell apoptosis. However, siRNA silencing of *PKC-α* resulted in a less significant apoptotic phenotype in comparison to that observed by miR-203 overexpression, thus suggesting that miR-203 may modulate multiple apoptotic genes that work together to regulate cell apoptosis [61]. Further studies must therefore be carried out to determine the additional apoptosis related targets of miR-203.

miR-143. miR-143 expression was reported to be downregulated in cancer tissues and inhibition of miR-143 promotes cell proliferation but hinders cell apoptosis. To determine the role that miR-143 plays in the apoptotic process, Akita (2002) investigated the possible targets of miR-143 and found that PKC-ε, a crucial enzyme in various cellular signaling pathways [62], was a putative target. Using the luciferase reporter assay it was determined that miR-143 specifically targets *PKC-ε*, and overexpression of miR-143 increases the cell apoptosis in A549 cells [63]. PKC-ε was suggested to play a role in regulating the antiapoptotic signaling pathway through the upregulation of Bcl-2 with a concurrent suppression of proapoptotic Bid [64–66]. Furthermore, PKC-ε is able to activate Akt to apply its prosurvival effects [67, 68]. Therefore, the targeting of *PKC-ε* could potentially be a valuable therapeutic strategy for lung cancer.

3.3. Other miRNAs

miR-198. miR-198 is downregulated in NSCLC cell lines and overexpression of this miRNA inhibits cell viability and enhances apoptosis in A549 cells. Overexpression of miR-198 induces the expression of poly(ADP-ribose) polymerase (PARP) and of cleaved caspase-3. miR-198 was also able to inhibit growth of tumor grafts in nude mouse. *FGFR1*, a lung cancer oncogene, which is a membrane-bound receptor tyrosine kinase that regulates proliferation via the MAPK and PI3K pathway, much like EGFR, was found to be a direct target of miR-198 [69].

miR-146a. Expression of miR-146a is low in malignant tissues in comparison to corresponding adjacent normal lung tissues. Functionally, miR-146a suppresses cell growth, inhibits cell migration and increases cellular apoptosis [70]. Upregulation of miR-146a expression via miR-146a mimic transfection resulted in the downregulation of EGFR as well as phosphorylated EGFR, both at the mRNA and at protein levels. Furthermore, downstream pathways (ERK-1/2, AKT, and STAT) were also downregulated in response to miR-146a mimic transfection, albeit with a weaker effect as that seen by cells transfected with *EGFR* specific siRNA. miR-146a mimic also led to the decrease of phosphorylation of the NF-κB inhibitor IκBα, but not total IκBα. Levels of phospho-NFκB, total NF-κB, and the total immune-modulating kinase, IRAK-1, were also found to be decreased following miR-146a mimic transfection, suggesting that miR-146a regulates NF-κB and IRAK-1 signaling [70].

miR-26a. miR-26a expression is downregulated in lung cancer tissues relative to normal tissues. Transfection of miR-26a into A549 cells was able to decrease cell proliferation, block the G1/S phase transition of cell cycle, and induce apoptosis [71]. The chromatin regulator enzyme EZH2, which regulates survival and metastasis of cancer cells [72], was found to be a direct target of miR-26a. Downregulation of *EZH2* expression, caused by overexpression of miR-26a will transactivate downstream tumor suppressor genes *DAB2IP* and *RUNX3*. DAB21P is a potent growth inhibitor that induces G0/G1 phase cell cycle arrest and could lead to apoptosis [73], while RUNX3 leads to cell cycle arrest, apoptosis, and significant decrease of tumor growth and abrogation of metastasis [74].

miR-451. Poor tumor differentiation, advance pathological state, lymph node metastasis, and poor prognosis are associated with downregulation of miR-451, which occurs in lung cancer [75]. To observe the functions of miR-451, Wang et al. (2011) upregulated miR-451 expression via mimics and observed suppressed *in vitro* proliferation, chromatin condensation and nuclear fragmentation upon 4′,6-diamidino-2-phenylindole (DAPI) staining, and significant caspase-3 activity. These results suggested that ectopic expression of miR-451 was able to induce an increase in apoptosis in a caspase-3 dependent manner. In addition, the *RAB14* gene was identified as a direct target of miR-451. Inhibition of *RAB14* led to a decrease in phosphorylation of Akt, which subsequently decreased levels of Bcl-2 protein expression

Table 1: Upregulated apoptosis-associated miRNAs in lung cancer.

MicroRNA	Target genes	Function	Cell lines	*In vivo* models	Citation
miR-197	BMF, NOXA	Repress *p53*-dependent apoptotic cascade miR inhibition decreases cell viability miR inhibition impairs cell growth and anchorage-independent colony formation	A549, Calu-1, NIH-H460, NCI-H1299	Nude mice	[35]
miR-21	SPRY1, SPRY2, BTG2, PDCD4, APAF1, FasL, RHOB	Enhance tumor proliferation and survival Inhibit apoptosis miR deletion suppresses Ras-driven transformation	N/T	K-ras^{LA2} mice CAG-miR-21 transgenic mice CAG-miR-21; K-ras^{LA2} compound mutant mice	[37]
miR-212	AChE	Prevent apoptosis Maintain cell proliferation capacity Modulate CDDP-induced NSCLC cell apoptosis	HEK-293T, NCI-H520, NCI-H460, SK-MES-1, BEAS-2B	Nude mice	[43]
miR-17-5p, miR-20a	E2F1	miR inhibition reduces of cell growth. miR inhibition induces apoptosis and increases proportions of sub-G1 populations.	Calu-6, A549, ACC-LC-172	N/T	[45]

N/D: not determined; N/T: not tested.

Table 2: Downregulated apoptosis-associated miRNAs in lung cancer.

MicroRNA	Target genes	Function	Cell lines	*In vivo* models	Citation
miR-7	BCL-2	Suppress cell proliferation and induce cell apoptosis Inhibit cancer cell migration *in vitro* Reduce tumorigenicity *in vivo*	A549, NCI-H1299, NCI-H1355, NCI-H460, MRC-5, HEK-293T	Nude mice	[51]
miR-198	FGFR1	Inhibit lung cancer cells proliferation Enhance cell apoptosis Inhibits growth of tumor graft in nude mouse	A549, NCI-H460	Athymic BALB/c nude mice.	[69]
miR-451	RAB14	Inhibit *in vitro* proliferation and enhance apoptosis Decrease phosphorylation of AKT and increased BAX or Bad protein level Associated with *in vivo* proliferation capacity	A549, SPC-A1, NCI-H520	Athymic BALB/c nude mice.	[75]
miR-192	RB1	Inhibit cell proliferation and promotes cell apoptosis Arrest cell in G1 phase Inhibit tumorigenesis *in vivo*	A549, NCI-H460, 95D	Athymic BALB/c nude mice.	[76]
miR-335	BCL-W, SP1	Suppress proliferation and invasion ability of cells Induce apoptosis Suppress metastasis and invasiveness of cells	A549, NCI-H1299	N/T	[52]
miR-608	N/D	Increase cell death in *Bcl-xL* silenced cells	A549, SK-LU-1	N/T	[55]
miR-203	PKCα	Decrease cell proliferation Promote cell apoptosis, but this effect only partially relies on its downregulation of PKCα	A549	N/T	[61]
miR-413	PKCε	Inhibit cell proliferation and enhance apoptosis	A549, Calu-1	N/T	[63]
miR-146a	EGFR	Inhibit cell growth and induces cell apoptosis Suppress motility Enhance cell proliferation inhibitory effect of TKIs and cetuximab	NCI-H358, NCI-H1650, NCI-H1975, NCI-H292, HCC827	N/T	[70]
miR-26a	EZH2	Inhibit cell proliferation *in vitro*Block G1/S phase transition and induced apoptosis Decreased metastasis capacity and invasion	SPC-A1, A549, SK-MES-1	N/T	[71]

N/D: not determined; N/T: not tested.

and increased proapoptotic Bax or Bad protein expression. As the expression levels of RAB14 protein were inversely correlated with the expression levels of miR-451 in NSCLC tissues it was concluded that downregulation of RAB14 may be the mechanism by which miR-451 carries out its tumor suppressor functions [75].

miR-192. miR-192 was found to be downregulated in A549, NCI-H460, and 95D cell lines [76]. Cell viability was greatly decreased following miR-192 upregulation, while levels of apoptosis were elevated with induced expression of PARP protein and cleaved caspase-7, thus suggesting that miR-192 induces apoptosis through the caspase pathway. Using bioinformatics analysis, *RB1* gene was determined to be a putative target of miR-192 and luciferase reporter assays confirmed direct binding of miR-192 to the 3′-UTR of this gene [76]. Since RB1 plays a vital role in regulating cell apoptosis, its downregulation was shown to induce γ-H2AX foci formation, a marker of DNA damage, and to promote apoptosis in A549 cells [77].

4. miRNA and Response to Cancer Therapy

Many cancer therapies available today aim to induce tumor-selective cell death; however resistance to chemotherapeutics is a significant obstacle to the long-term treatment and survival of NSCLC patients [78]. Presently, there are various chemotherapeutics that are being utilized in the treatment of lung cancer, including FDA approved drugs (DDP, paclitaxel, docetaxel, gemcitabine, and EGFR-TKIs), natural compounds (curcumin), and small organic compounds (PRIMA-1) (see Table 3). The association of miRNAs as regulators of malignancy and apoptosis has been widely reported; thus it is reasonable to assume that miRNAs play significant roles in sensitivity/resistance to common cancer treatments (see Figure 2) [79]. Indeed, recent studies have demonstrated miRNAs as potential agents involved in the sensitivity of lung cancer cells to cytotoxic therapy.

4.1. Cisplatin- (DDP-) Related miRNAs. DDP is a platinum-coordinated complex that is the most widely used chemotherapy for human NSCLC in the past two decades [80–82]. However, multiple administration of DDP results in the development of drug resistance leading to failure of treatment, as demonstrated by tumor growth or tumor relapse [78, 83]. Therefore, to overcome the treatment plateau of DDP on NSCLC, the biological mechanisms by which DDP action is enforced must be further elucidated. As miRNAs act as critical regulators in the development of drug resistance, it would be interesting to research the mechanism through which oncogenic miRNAs modulates DDP-induced apoptosis in NSCLC.

miR-451. miR-451 was downregulated in NSCLC tissues in comparison to normal lung tissues, and upregulation of miR-451 enhances DDP chemosensitivity in A549 cells by inhibiting cell growth and inducing apoptosis enhancement [84]. Bian et al. (2011) demonstrated in their study that upregulation of miR-451 enhanced caspase-3-dependent apoptosis through the inactivation of the Akt signaling pathway, which in turn decreased Bcl-2 while increasing expression of Bax protein levels. Furthermore, results of Annexin V-FITC apoptosis assay indicated that in miR-451 transfected A549 cells (A549/miR-451) a higher percentage of apoptosis was observed in comparison to mock A549 cells. Caspase-3 activity in A549/miR-451 treated with DDP was significantly increased against the control, thus suggesting that miR-451 upregulation increases chemosensitivity of A549 cells by enhancing DDP-induced apoptosis. Together these results suggest a possible strategy for treatment of human NSCLC through the combined application of DDP treatment with miR-451 upregulation [84].

miR-31. On the other hand, miR-31 is upregulated in NSCLC cell lines and was demonstrated to induce DDP resistance. To demonstrate this, Glavinas et al. (2004) transfected miR-31 mimics into DDP-sensitive SPC-A-1 cells which led to a marked increase in the resistance of SPC-A-1 cells, while transfection of miR-31 inhibitors increased sensitivity of resistant NCI-H1299 to DDP treatment. To elucidate the mechanism by which DDP resistance is induced by miR-31, bioinformatics analysis was carried out and *ABCB9*, a membrane transporter involved in drug uptake [85], was predicted to be a target gene. The luciferase reporter assay then confirmed direct miR-31 regulation of *ABCB9* by binding to its 3′UTR [86]. Overexpression/knockdown studies indicated a significant decrease in the percentage of DDP-induced apoptotic cells when miR-31 was increased via mimics and a marked increase in DDP-induced apoptotic cells when miR-31 inhibitors were introduced, thus suggesting that miR-31 exerts an antiapoptotic effect in DDP-induced apoptosis through the inhibition of *ABCB9*.

4.2. Paclitaxel-Related miRNAs. Paclitaxel was the first identified member of taxanes in the list of FDA-approved anticancer drugs. This compound has been shown to have significant single-agent activity against various solid tumors [87, 88] including NSCLC [89]. However, combination of this compound with DDP or carboplatin showed superior response and improved survival rates [90].

miR-133a/b and miR-361-3p. High-throughput screening (HTS) approach was performed by Du and colleagues in 2013 to identify miRNAs that modulate lung cancer cell survival and response to paclitaxel treatment [91]. Using three NSCLC cell lines that have distinct genetic backgrounds (NCI-H1155, NCI-H1993, and NCI-H358), inhibition of two miRNAs (miR-133a/b and miR-361-3p) was found to potently decrease cell viability, although cytotoxicity of the two miRNAs vary greatly, which may be due to different endogenous expression levels of the miRNAs in each cell line. Interestingly, the inhibitors of miR-133a/b and miR-361-3p were found to reduce cell survival through different mechanisms. miR-133a/b inhibitor was able to dramatically increase apoptotic events as seen by increased percentage

Table 3: Drug-associated miRNAs in lung cancer.

Drug	MicroRNA	Target genes	Function	Cell Lines	*In vivo* models	Citation
Docetaxel	miR-100	Plk1	Chemosensitize lung adenocarcinoma cells to docetaxel Suppress cell proliferation, enhance apoptosis and cell cycle arrest in the G2/M phase of cell cycle Down-regulate *in vivo* cell proliferating ability	SPC-A1, A549, NCI-H1299, SPC-A1/DTX	Nude mice	[102]
	miR-650	ING4	Confer docetaxel chemoresistance both *in vitro* and *in vivo*	SPC-A1, NCI-H1299	Athymic BALB/c nude mice	[105]
Cisplatin	miR-451	N/D	Enhance DDP chemosensitivity Inhibit growth and enhance apoptosis	A549	BALB/c nude mice	[84]
	miR-31	ABCB9	Induced DDP resistance Antiapoptotic effect	SPC-A1, LTEP-A2, NCI-H460, NCI-H1299	N/T	[86]
Paclitaxel	miR-133a/b, miR-361-3p	N/D	Oncogenic miR inhibition reduces cell survival	NCI-H1155, NCI-H1993, NCI-H358	N/T	[91]
	miR-101	EZH2	Decrease proliferation and invasive ability of cells Sensitize cells to paclitaxel	NCI-H226, A549, NCI-H358, 801D	N/T	[98]
Gemcitabine	miR-133b	MCL-1, BCL-W	Increase apoptosis Gemcitabine sensitivity	A549, NCI-H23, NCI-H2172, NCI-H226, NCI-H522, NCI-H2009, NCI-H1703	N/T	[111]
EGFR-TKI	miR-30b/c miR-221/222	APAF-1, BIM	Gefitinib-induced PARP cleavage Decrease cell viability Restore gefitinib sensitivity	Calu-1, A549, PC-9, HCC827	N/T	[123]
	miR-214	PTEN	Oncogenic Knockdown sensitizes cells to gefitinib	HCC827, HCC827/GR	N/T	[126]
	miR-133b	EGFR	Inhibit cell's growth and invasion abilities Enhance EGFR-TKI sensitivity	PC-9, A549, NCI-H1650, NCI-H1975	N/T	[131]
Curcumin	miRNA-186*	Caspase-10	Inhibits cell apoptosis Down-regulation of miR-186 by curcumin induces apoptosis	A549, A549/DDP	N/T	[154, 157]
TRAIL	miR-221, miR-222	N/D	Impair TRAIL-dependent apoptosis Induce TRAIL-resistance	Calu-1, A549, NCI-H460	N/T	[168]
	miR-34a, miR-34c	PDGFR-α, PDGFR-β	Augment TRAIL response Reduce migratory and invasive capacity of cells	NCI-H460, A549, NCI-H1299, Calu-6 NCI-H1703,	N/T	[169]
	miR-212	PED	Increase sensitivity to TRAIL Tumor suppressor	Calu-1, NCI-H460	N/T	[175]
PRIMA-1	miR-34a	N/D	Induce apoptosis in the lung cancer cells containing mutant *p53*	A549, NCI-H211, NCI-H1155, NCI-H1299	N/T	[148]
Multidrug Resistance	miR-200bc/429	BCL2, XIAP	Enhanced sensitivity to various anticancer drugs including VCR, CDDP, VP-16, and ADR	A549, A549/CDDP	N/T	[158]
	miR-181b	BCL2	Increased sensitivity to a number of anticancer drugs including VCR, 5-Fu, CDDP, VP-16, and ADR	A549, A549/CDDP	N/T	[161]

N/D: Not determined, N/T: Not tested.

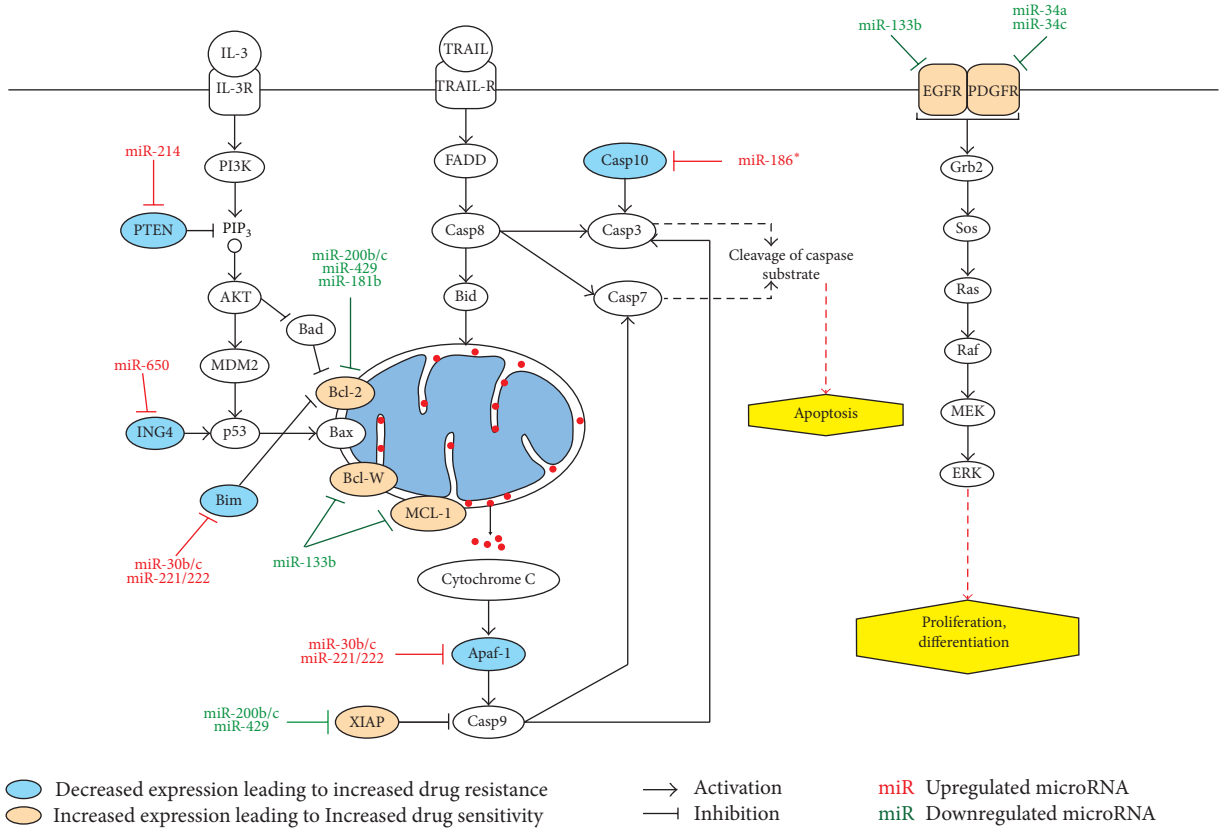

Figure 2: Scheme depicting the roles miRNAs play in sensitivity and resistance to common cancer treatments.

of cells undergoing apoptosis and increased levels of activated caspase-3. However miR-361-3p only showed a modest effect on caspase-3 activation thus suggesting that additional mechanisms are involved in the cytotoxicity of this miRNA. The effect of miRNA inhibitors on cell cycle distribution was then evaluated and results indicated that S phase arrest contributes to cytotoxicity induced by miR-133a/b and miR-361-3p inhibitors. Together these results suggest that miR-133a/b and miR-361-3p may function as oncogenes in cancer cells by regulating tumor suppressor genes.

miR-101. Increasing evidence has revealed that EZH2 has oncogenic properties, as an increased expression of EZH2 augments proliferation and invasion of cancer cells [92–94], while depletion leads to a decline in cell proliferation, increased apoptosis, and inhibition of metastatic tumor growth *in vivo* [95, 96]. Overexpression of EZH2 has been associated with tumor progression and cancer aggressiveness in NSCLC [97]. In a study by Zhang and colleagues (2011), it was discovered that a decreased expression of miR-101 was associated with EZH2 overexpression in NSCLC tissues [98]. Luciferase reporter assay revealed that miR-101 regulates EZH2 expression through the binding of its 3′UTR mRNA. Overexpression of miR-101 led to a decrease in EZH2 protein levels with subsequent decrease in the proliferation and invasive ability of NSCLC cells. Furthermore, overexpression of miR-101 led to a sensitization of NSCLC cells to paclitaxel.

4.3. Docetaxel-Related miRNAs. Docetaxel, a semisynthetic analog of paclitaxel, is one of the first-line chemotherapy regimens for advanced NSCLC, with genotoxic effects caused by microtubule stabilizing, apoptotic induction through microtubule bundling, and Bcl-2 blocking [99, 100].

miR-100. In a miRNA microarray profiling carried out by Rui and colleagues in 2010, miR-100 was significantly downregulated in docetaxel-resistant SPC-A1/DTX cells relative to SPC-A1 parental cells [101]. To elucidate the role that miR-100 plays in the formation of docetaxel resistance, the authors' transfected miR-100 mimics SPC-A1/DTX cells [102]. Results suggested that restoration of miR-100 expression chemosensitizes cells to docetaxel *in vitro*, complemented with a suppression of cell proliferation, enhancement of apoptosis, and cell cycle arrest in the G2/M phase of cell cycle. Ectopic miR-100 expression was also able to downregulate *in vivo* cell proliferating ability. Moreover, *PLK1* gene was identified to be a direct target of miR-100. *PLK1* plays a role in promotion of cell proliferation and overexpression of this gene has been observed in various human cancers [103] including NSCLC [104]. Knockdown of Plk1 protein expression by miR-100 led to a significant suppression of cell proliferation of SPC-A1/DTX, dramatic increase of early apoptosis rate, G2/M arresting population, and an increase in the response of SPC-A1/DTX cells to docetaxel both *in vitro* and *in vivo*. miR-100 was therefore concluded to function as a chemosensitizer

restorer to docetaxel by targeting *PLK1* and inducing the suppression of cell proliferation, enhancement of apoptosis, and mitotic arrest.

miR-650. High expression of miR-650 can be found in lung cancer tissues, and its dysregulation is correlated with advance clinical stage as a poor prognostic factor for these patients [105]. Furthermore, Huang et al. (2013) determined that the expression of miR-650 is negatively correlated with patients' response to docetaxel. Using two docetaxel-resistant cell lines (SPC-A1/DTX and H1299/DTX), the authors demonstrated that downregulation of miR-650 was able to reverse the resistance. *ING4*, a novel tumor suppressor gene, was then identified as the functional target of miR-650 and results from flow cytometry and Hoechst staining assays indicated that miR-650 inhibitor was able to induce an increase in caspase-3-dependent apoptosis. Cells transfected miR-650 inhibitors exhibited decreased expression of Bcl-2 protein, with an increased expression of Bax protein, led to the progression of apoptosis. The findings of this study confirmed that miR-650 was able to confer docetaxel chemoresistance through the regulation of Bcl-2/Bax expression by targeting of *ING4* [105].

4.4. Gemcitabine-Related miRNA. Gemcitabine, a pyrimidine nucleoside antimetabolite, has been shown to be an effective agent most particularly when administered in combination regimes [106]. Due to its theoretical ability of interfering with the inhibition of repair of platinum-induced DNA damage, gemcitabine is the perfect partner for platinum compounds. Gemcitabine in combination with DDP represents a common first-line treatment for patients with advanced NSCLC, especially in Europe [80, 107–110].

miR-133b. miR-133b is greatly reduced in cancer tissue in comparison to adjacent normal lung tissue [111]. Prediction programs identified two common predicted targets of miR-133b, the antiapoptotic *MCL-1* and *BCL-W*, both of which are members of the antiapoptotic *BCL-2* family [112] and have previously been reported to be increased in both solid and hematological malignancies including lung cancer [113, 114]. Transfection of miR-133b using pre-miR-133b resulted in a decrease in Bcl-W and Mcl-1 protein expression with a moderate increase of apoptosis. However combination treatment of miR-133b overexpression with 24 hours treatment of gemcitabine resulted in a greater degree of cleaved PARP expression as well as apoptosis. This concludes that miR-133b is able to target prosurvival molecules and induce apoptosis in the setting of chemotherapeutic agents [111].

4.5. Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitors- (EGFR-TKIs-) Related miRNAs. EGFR is a plasma membrane glycoprotein that belongs to a family of four different tyrosine kinase receptors (EGFR (ErbB1), HER2/neu (ErbB2), HER3 (ErbB3), and HER4 (ErbB4)) [115]. Dimerization of EGFR may result in cancer cell proliferation, inhibition of apoptosis, invasion, metastasis, and tumor induced neovascularization [116]. Mutations and subsequent overexpression of EGFR can be found in all histologic subtypes of NSCLC [117]. Deletion in exon 19, which removes the conserved sequence LREA, and a single point mutation in exon 21, which leads to the substitution of arginine for leucine at position 858 (L858R), are the most clinically relevant and extensively studied drug-sensitive mutations [118]. Studies have shown that these mutations preferentially bind to first generation EGFR-TKIs, gefitinib and erlotinib [119, 120]. First generation EGFR-TKIs function by selectively targeting the receptor via a competitive, reversible binding at the tyrosine kinase domain, thus leading to the inhibition of ATP binding and subsequent signal transduction and downstream functions [121]. However, acquired resistance to EGFR-TKIs in the metastatic setting is unavoidable. While the average progression-free survival (PFS) is between 10 and 16 months, treatment duration can last as short as 1 month [122]. Drug resistance therefore still remains a problem and new therapies and strategies must be developed to overcome such resistance.

miR-30b/c and miR-221/222. EGF and MET receptors control gefitinib-induced apoptosis and NSCLC tumorigenesis through the downregulation of specific oncogenic miRNAs, miR-30b/c, and miR-221/222 [123]. Using bioinformatics analysis and luciferase assays, *APAF-1* and *BIM* (previously found to play a role in TKI sensitivity [124, 125]) were determined to be direct targets of miR-221/222 and -30b/c. To investigate the roles these miRNAs play in gefitinib-induced apoptosis, wild-type EGFR expressing NSCLC cells (Calu-1 and A549) and cells with EGFR exon-19 deletions (PC9 and HCC827) were utilized. Upon gefitinib treatment, significant downregulation of miR-30b/c and miR-221/222 with an increased BIM and APAF-1 protein levels were observed only in PC9 and HCC827 sensitive cells. To further determine the contribution of miR-30b/c and miR-221/222-mediated APAF-1 and BIM downregulation to cellular TKI response, Garofalo et al. (2012) overexpressed APAF-1 and BIM in A549 resistant cells, which consequently led to gefitinib-induced PARP cleavage. Furthermore, as miR-30b/c and miR-221/222 are regulated by MET, a strong downregulation was observed of these miRNAs when Calu-1- and A549-MET overexpressing cells were treated with MET inhibitors SU11274. Furthermore an increase in caspase-3/7 activity and decreased cell viability was observed in SU11274-treated Calu-1 cells following exposure to varying gefitinib concentrations. Together, these results suggest that MET inhibition restores gefitinib sensitivity in TKI-resistant Calu-1 through downregulation of miR-30b/c and miR-221/222 [123].

miR-214. miR-214 is significantly upregulated in gefitinib resistant lung adenocarcinoma cell line, HCC827/GR, in comparison to parental HCC827 lung adenocarcinoma cells. HCC827/GR was obtained by exposing HCC827 cells to increasing concentrations of gefitinib over six months [126]. Using dual-luciferase reporter assay, Wang et al. (2012) confirmed PTEN as a direct functional target of miR-214. PTEN encodes a 403 amino acid dual-specificity lipid and protein phosphatase which functions as a tumor suppressor in many tumors [127, 128]. Knockdown of miR-214 expression

resulted in the upregulation of PTEN protein and inactivation of AKT, which is largely linked to antiapoptotic function [129, 130]. Furthermore, knockdown of miR-214 resensitized HCC827/GR to gefitinib, as demonstrated through MTS assay. miR-214 was thus concluded to potentially serve as a therapeutic target to reverse the acquired resistance of gefitinib in lung adenocarcinoma cells.

miR-133b. Expression of miR-133b is significantly downregulated in NSCLC tissues in comparison to nonneoplastic lung tissues [131], and the 3′UTR of *EGFR* was found to be a direct target of this miRNA thus inhibiting its expression. Treatment of EGFR-addicted lung cancer cells, PC-9 and A549 with miR-133b mimic inhibited phosphorylation of EGFR, AKT, and extracellular signal-related kinase (ERK)1/2, thus inhibiting their growth and invasion abilities. However in non-EGFR-addicted NSCLC cells NCI-H1650 and NCI-H1975, no significant changes in the expression of phosphorylated EGFR, AKT, and ERK1/2 were found. Furthermore, miR-133b was able to restore or enhance EGFR-TKI sensitivity in NSCLC cells, especially in EGFR-addicted cells. These findings reveal that transfection of miR-133b in EGFR-addicted NSCLC has the therapeutic potential for overcoming EGFR-TKI resistance [131].

4.6. PRIMA-1-Related miRNA. The tumor suppressor *p53* gene regulates cell growth through the activation of the transcription of numerous genes specifically those involved in cell cycle regulation, apoptosis, and genomic stability [132–134] andhas also been implicated in the response to anticancer therapies [133]. *p53* has been reported to be frequently mutated in humans cancers with mutations occurring in greater than 50% of lung tumors [135, 136]. Restoration of wild-type p53 function has led to regression of cancers in mice [137, 138], and thus efforts to treat cancers through the reactivation of *p53* with a low-molecular-weight compound such as PRIMA-1 (*p53*-dependent reactivation and induction of massive apoptosis) [139, 140] are widely supported.

miR-34a. In a study conducted by Duan et al. (2010), the role of miR-34 family members in regulating PRIMA-1 induced apoptosis was investigated. The authors discovered that PRIMA-1 was able to upregulate miR-34a in *p53* mutant cells. Previous studies have shown evidence that the miR-34 family plays a role in the regulation of cell proliferation and apoptosis [19, 141–147]. The results of this study suggest that PRIMA-1 is able to restore wild-type function to mutant *p53*, which will upregulate miR-34a to induce apoptosis in lung cancer cells [148].

4.7. Curcumin Related miRNA. Curcumin is a compound extracted from the rhizomes of *Curcuma longa* L. and studies carried out exhibited its diverse pharmacological effects which include anti-inflammatory, antioxidant, and antitumor activities [149]. Previous studies have also shown that curcumin can induce apoptosis in many types of cancer cells [150, 151], through the inhibition of *NF-κB*, *survivin/BIRC5,* and *BCL-2* [152, 153]. However few studies have been carried out to report the importance of miRNA expression modulation in mediating the biological effects of curcumin.

miRNA-186*. In a study conducted by Zhang et al.(2010), curcumin was shown to have the ability to inhibit cell proliferation and induce apoptosis in A549 cells. The authors performed a cluster analysis on the expression profiles on curcumin-treated and dimethyl sulfoxide (DMSO) control-treated samples and found that miR-186* was shown to be significantly downregulated in response to curcumin treatment, thus suggesting that miR-186* may play an oncogenic role in human lung cancer cells. Inhibition of miR-186* was shown to greatly decrease cell proliferation in A549 cells and increase the induction of apoptosis. Furthermore, caspase-10 was revealed to be a direct target of miR-186 [154]. This study thus provided the first evidence that miR-186* is essential for the anticancer effects of curcumin in A549 cells and that caspase-10 may be an important target of miR-186* in preventing apoptosis.

However, even though curcumin has exhibited antitumor activity, there has been concern regarding the effects of curcumin on multidrug resistant cells [155, 156]. To analyze such effects, A549/DDP, the DDP-resistant derivative of parental A549 cells generated by coculturing parental A549 cells with 6 nm DDP to maintain the drug resistance phenotype, was utilized. In a study conducted by Zhang et al. (2010), a comprehensive miRNA profiling of untreated multidrug-resistant cell line (A549/DDP) was performed and compared against results obtained for A549/DDP cells treated with curcumin. Results showed that miR-186* was downregulated more than 2.5-fold compared to levels in control cells. The antiapoptotic effects of miR-186* in A549/DDP cells were investigated and it was found that transfection of miR-186* mimics led to an inhibition of apoptosis in comparison to that in the control, thus suggesting that miR-186* plays an oncogenic role in this cell line. To confirm the role miR-186* plays in curcumin-induced A549/DDP apoptosis, flow cytometry was used to detect the rate of apoptosis in A549/DDP cells treated with curcumin, control cells, or curcumin combined with miR-186* mimic cells. Results indicated that apoptosis in the combination group was significantly decreased in comparison to cells treated with curcumin [157]. These findings reveal that curcumin is able to induce apoptosis in the multidrug resistant cell line by downregulating miR-186*.

4.8. Multidrug Resistance

miR-200bc/429. In 2012, Zhu et al. reported that the miR-200bc/429 cluster was downregulated in multidrug-resistant A549/DDP cells, in comparison to parental A549 cell [158]. Recent studies have suggested that aberrant DNA methylation of the promoter region of the miR-200bc/429 cluster may be a critical mechanism leading to dysregulated expression level of the miR-200 family [159, 160]. While the roles of the two sequence clusters of miR-200 family on the epithelial-to-mesenchymal transition of tumor cells are well studied, the role that this miRNA family plays on apoptosis has been minimally studied. Zhu et al. demonstrated using MTT that transfection of miR-200bc/429 cluster mimics

into A549/DDP greatly enhanced sensitivity of this cell line to various anticancer drugs including vincristine (VCR), etoposide (VP-16), adriamycin (ADR), and DDP. It was found that miR-200bc/429 cluster was able to modulate multidrug resistance (MDR) in lung cancer cell lines, at least in part by inhibiting the antiapoptotic Bcl-2 and XIAP protein expression, thus affecting the mitochondrial release of cytochrome c. Therapeutic methods that target the miR-200bc/429 clusters thus provides a promising method to enhance treatment effect of NSCLC.

miR-181b. In another study by Zhu et al. (2010), miR-181b was also found to be downregulated in multidrug-resistant A549/DDP cells, in comparison to parental A549 cell line [161]. To determine whether miR-181b has a direct role in MDR development, MTT assay was performed revealing that all A549/DDP cells transfected with miR-181b mimic exhibited a significant increase in sensitivity to a number of anticancer drugs including 5-fluorouracil (5-Fu), VCR, DDP, VP-16, and ADR. Bioinformatics analysis predicted antiapoptotic *BCL-2* as a potential target of miR-181, with two conserved target sites in the 3′UT region. Transfection of miR-181a in A549/DDP cells led to a significant decrease in Bcl-2 protein levels, as demonstrated by Western blot. Furthermore, A549/DDP miR-181b transfected cells also led to an increase in apoptosis as detected by flow cytometry. Together these results demonstrate miR-181b's ability to modulate the development of MDR in lung cancer cell lines, at least in part, by modulation of apoptosis through the targeting of the antiapoptotic *BCL-2*.

4.9. TRAIL-Related miRNAs. The Apo2L/tumor necrosis factor- (TNF-) α-related apoptosis inducing ligand (TRAIL) is a member of the TNF family that is known to induce apoptosis in various cancers [162]. Treatment of transformed cells with TRAIL has been shown to successfully induce apoptosis both *in vitro* and *in vivo* [162, 163]; however a wide range of human cancer cells are resistant to TRAIL-induced apoptosis [164].

miR-221 and -222. To identify the mechanisms by which miRNAs may play a role in TRAIL resistance, Garofalo et al. (2008) carried out a genome wide profiling of miRNAs in three different lung cancer cell lines (A459, Calu-1, and NCI-H460) and found that miR-221 and miR-222 were markedly upregulated in TRAIL-resistant cells. In TRAIL sensitive cells NCI-H460, TRAIL was able to induce the activation of the caspase cascade, evaluated by the appearance of cleaved fragments. However, transfection of NCI-H460 cells with pre-miRs-221 and -222 caused a significant reduction of TRAIL-mediated cell death machinery activation. Further experiments deduced that miR-221 and -222 directly targeted $p27^{Kip1}$, and inhibition of $p27^{Kip1}$ via pre-miR-221 and -222 transfection led to an increase in cell resistance to TRAIL as assessed by Annexin V staining, and PARP and caspase-8 activation. Taken together, the authors' results demonstrate that increased levels of miR-221 and -222 may modulate sensitivity of NSCLC cells to TRAIL with important implications in the design of new therapeutic agents.

miR-34a and miR-34c. In another study, miR-34a and miR-34c expression were found to be significantly downregulated in NSCLC cells and lung tumors in comparison to normal lung tissues. Performing a bioinformatics search, Garofalo et al. (2013) determined that *PDGFR-α* and *PDGFR-β* were targets of these miRNAs; both of which have been reported to be overexpressed and associated with poor outcome in lung cancer [165]. Through targeting *PDGFR-α* and *PDGFR-β*, miR-34a/c were able to decrease invasiveness as well as increase TRAIL-induced apoptosis. TRAIL resistance is common in lung tumors and it has been reported that PDGFR-α and PDGFR-β regulate the PI3K/Akt and ERK1/2 pathways [166, 167], which play a role in TRAIL-induced apoptosis [168]. Phosphorylation levels of ERKs were found to be decreased following ectopic expression of miR-34a/c; additionally caspase-3/7 assay revealed an increase in TRAIL sensitivity. This study demonstrates that inhibition of *PDGFR-α* and *PDGFR-β* by miR-34a/c is able to antagonize tumorigenicity and increase sensitivity to TRAIL-induced cell death [169].

miR-212. PED/PEA-15 is a death effector domain (DED) family member, which has been implicated in the processes of cell growth and metabolism [170–172]. Furthermore, PED/PEA-15 has a broad range of antiapoptotic ability, being able to inhibit both the intrinsic and extrinsic apoptotic pathways [171, 173]. Zanca and colleagues (2008) reported that PED/PEA-15 overexpression plays a role in TRAIL resistance in NSCLC [174]; however the mechanism that regulates its expression is not well known. In further studies, Incoronato et al. (2010) reported that NSCLC-affected lung tissue has an increased expression of PED/PEA-15 with a concurrent downregulation of miR-212 and decreased response to TRAIL treatment [175]. miR-212 negatively regulates *PED/PEA-15* by directly binding to its 3′UTR. miR-212 downregulation has previously been reported to be involved in lung cancer response to chemotherapy, in particular to docetaxel [101]. In this study, transfection of NSCLC Calu-1 cells with pre-miR-212 led to a decrease in PED/PEA-15 expression with increased caspase-8 activation following treatment with TRAIL, indicating increased sensitivity of Calu-1 cells to TRAIL-mediated cell death. Therefore, the expression of miR-212 could be used to predict therapeutic response to TRAIL in lung cancer.

5. Conclusions

In terms of molecular events occurring in tumors, evasion of apoptosis is an important hallmark of tumor progression. Recent evidence has exhibited deregulated miRNAs to play a role in the apoptotic process. In lung cancer, upregulated miRNAs have been shown to serve as oncogenes, targeting tumor suppressor, and/or proapoptotic genes, while downregulated miRNAs can function as tumor suppressors, targeting oncogenic and/or antiapoptotic genes. Additionally, studies have also indicated that miRNAs play a significant role

in altering sensitivity and resistance to cytotoxic treatment. Targeting of specific miRNAs could therefore potentially be used as valuable therapeutics for lung cancer. Together, these studies have illustrated the importance for further studies and validation of miRNAs and their targets. Furthermore, there is a serious shortage in research being carried out in miRNA-regulated apoptosis in SCLC. As SCLC accounts for 16.8% of lung cancer incidence and is a highly aggressive form of lung cancer it would be of great interest to determine the functions of miRNAs in regulation of apoptosis in this lung cancer subtype.

Abbreviations

γ-H2AX: Gamma, H2A histone family, member X
3′UTR: 3′-Untranslated region
5-Fu: 5-Fluorouracil
ABCB9: ATP-binding cassette, subfamily B (MDR/TAP), member 9
AChE: Acetylcholinesterase
ADR: Adriamycin
Akt: Protein kinase B
Apaf-1: Apoptotic peptidase activating factor 1
BAK: BCL2-antagonist/killer 1
BAX: BCL2-associated X protein
BCL-2: B-cell CLL/Lymphoma
BCL-W: BCL2-like 2
BCL-XL: BCL2-like 1
BIM: BCL2-like 11 (apoptosis facilitator)
BIRC5: Baculoviral IAP repeat containing 5
BMF: Bcl-2-modifying factor
BTG2: BTG family, member 2
DAB2IP: Disabled homolog 2-interacting protein
DDP: Cisplatin
DMSO: Dimethyl sulfoxide
E2F1: E2F transcription factor 1
EGFR: Epidermal growth factor receptor
ERK1: Mitogen-activated protein kinase 3
ERK2: Mitogen-activated protein kinase 1
EZH2: Histone-lysine N-methyltransferase
FasL: Fas ligand
FDA: Food and drug administration
FGFR1: Fibroblast growth factor receptor 1
FITC: Fluorescein isothiocyanate
HTS: High-throughput screening
IκBα: Nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor alpha
ING4: Inhibitor of growth family, member 4
IRAK-1: Interleukin-1 receptor-associated kinase 1
MAPK: Mitogen activated protein kinase
MCL1: Molecule myeloid leukemia 1
MDR: Multidrug resistance
miRNA: MicroRNA
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells
NSCLC: Non-small cell lung cancer
NOXA: Phorbol-12-myristate-13-acetate-induced protein 1
$p27^{Kip1}$: Cyclin-dependent kinase inhibitor 1B
p53: Tumor protein p53
PDCD4: Programmed cell death 4 (neoplastic transformation inhibitor)
PDGFR-α: Platelet-derived growth factor receptor-alpha polypeptide
PDGFR-β: Platelet-derived growth factor receptor-beta polypeptide
PEA-15: Astrocytic phosphoprotein PEA-15
PED: Preimplantation embryonic development
PI3K/AKT: Phosphatidylinositol 3-kinase/protein kinase B
PKC: Protein kinase C
PKC-α: Protein kinase C-alpha
PKC-ε: Protein kinase C-epsilon
PLK1: Polo-like kinase 1
PRIMA-1: *p53*-dependent reactivation and induction of massive apoptosis
PFS: progression-free survival
PUMA: BCL2 binding component 3
RAB14: RAB14, member RAS oncogene family
RB1: Retinoblastoma 1
RHOB: Ras homolog family member B
RUNX3: Runt-related transcription factor 3
SCLC: Small cell lung cancer
SP1: Specificity Protein 1
SPRY1: Sprouty homolog 1, antagonist of FGF signaling (*Drosophila*)
SPRY2: Sprouty homolog 2 (*Drosophila*)
STAT: Signal transducer and activator of transcription
TRAIL: Apo2L/tumor necrosis factor- (TNF-) α-related apoptosis inducing ligand
TGF-β: Transforming growth factor-beta
VCR: Vincristine
VP-16: Etoposide
WNT: Wingless-type MMTV integration site family
XIAP: X-linked inhibitor of apoptosis.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Estrogen-Related Receptor Alpha Confers Methotrexate Resistance via Attenuation of Reactive Oxygen Species Production and P53 Mediated Apoptosis in Osteosarcoma Cells

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Osteosarcoma (OS) is a malignant tumor mainly occurring in children and adolescents. Methotrexate (MTX), a chemotherapy agent, is widely used in treating OS. However, treatment failures are common due to acquired chemoresistance, for which the underlying molecular mechanisms are still unclear. In this study, we report that overexpression of estrogen-related receptor alpha (ERRα), an orphan nuclear receptor, promoted cell survival and blocked MTX-induced cell death in U2OS cells. We showed that MTX induced ROS production in MTX-sensitive U2OS cells while ERRα effectively blocked the ROS production and ROS associated cell apoptosis. Our further studies demonstrated that ERRα suppressed ROS induction of tumor suppressor P53 and its target genes NOXA and XAF1 which are mediators of P53-dependent apoptosis. In conclusion, this study demonstrated that ERRα plays an important role in the development of MTX resistance through blocking MTX-induced ROS production and attenuating the activation of p53 mediated apoptosis signaling pathway, and points to ERRα as a novel target for improving osteosarcoma therapy.

1. Introduction

Osteosarcoma (OS) is one of the most common forms of childhood and adolescent cancer, comprising approximately 20% of all primary bone cancers, which is the second leading cause of mortality in this age group [1, 2]. Currently, the use of neoadjuvant chemotherapy combined with surgical resection is the mainstay therapy in OS [3]. The antifolate, chemotherapeutic agent methotrexate (MTX), is widely used to treat OS. However, approximately 30% of the OS patients suffer the recurrent or metastatic diseases due to the development of drug resistance [4]. Therefore, it is imperative to understand the molecular mechanisms underlying the MTX resistance and identify novel treatment strategies for this disease.

Estrogen-related receptors alpha (ERRα) is an orphan nuclear receptor that plays a key role in regulating metabolic processes. ERRα is abundantly expressed in tissues with high-energy demand such as heart, kidney, skeletal muscle, and brown adipose tissues [5, 6]. In addition to its role in the control of energy metabolic processes, there is cumulative evidence showing potential functions of ERRα in cancer development and progress. ERRα overexpression is associated with poor clinical outcomes not only in breast, ovarian, and prostate cancers, but also in nonendocrine related colon cancer [7–10]. Our previous reports have shown that ERRα suppression efficiently induced cancer cell death via induction of reactive oxygen species (ROS) production [11, 12]. ROS are chemically reactive molecules and have important roles in cell signaling and homeostasis [13]. ROS level can be dramatically increased as a response to different stress conditions, which can lead to several biological effects, ranging from alterations in cell signaling pathway and

gene expression to mutagenesis, mitogenesis, or apoptosis [14, 15]. Recent studies demonstrated that chemotherapeutic drugs including MTX can induce cell death by promoting ROS production [16, 17]. We thus hypothesized that ERRα may be involved in chemotherapy resistance in osteosarcoma.

In this study, we demonstrated that elevated ERRα can result in the development of MTX resistance through blocking MTX-induced ROS production and attenuating p53-dependent apoptosis in osteosarcoma cells. Our study suggests ERRα is a novel target for improving osteosarcoma therapy.

2. Materials and Methods

2.1. Reagents and Antibodies. Methotrexate (MTX), N-acetylcysteine (NAC), and hydrogen peroxide (H_2O_2) were purchased from Sigma Chemical Company (St. Louis, MO, USA). For lentivirus generation, pMD2.G (envelope plasmid) and psPAX2 (packaging plasmid) vectors from Addgene (Cambridge, MA, USA) were used. P53 antibody was purchased from Biochem; cleaved PARP and cleaved caspase 7 antibodies were from Cell Signaling Technology (Boston, MA, USA).

2.2. Cell Culture and Generation of Stable Sublines. U2OS parental cells and human embryonic kidney (HEK 293T) cells were cultured in Dulbecco's Modified Essential Medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (Gemini Bio Products, West Sacramento, CA, USA) at 37°C under 5% CO_2.

To generate ERRα overexpression U2OS stable sublines, full length cDNA of ERRα was cloned into the expression vector pLenti4/V5-DEST (Invitrogen). Lentiviruses were produced from these constructs using a three-plasmid packaging system as described [18]. U2OS cells were infected with the lentiviruses and selected with blasticidin (2.5 μg/mL, Invivogen, San Diego, CA) for 4–6 weeks. Individual blasticidin-resistant clones were isolated and assayed for ERRα expression by immunoblotting. Clones homogeneously expressing ERRα were maintained in DMEM supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum and 1 μg/mL blasticidin at 37°C under 5% CO_2. All cell lines were used within 20 passages.

2.3. Cell Survival Rate Assay. Cells in logarithmic growth phase were seeded in 6-well plates at a density of 100,000 cells per well and treated with different concentrations of MTX (0-1 μM). Cells were harvested after 24, 48, and 72 hours and counted by Beckman Z1 Coulter Counter. Each treatment was performed in triplicate wells at least 3 times.

2.4. Trypan Blue Exclusion Assay. Cells in logarithmic growth phase were seeded in 6-well plates at a density of 100,000 cells per well and treated with different concentrations of MTX (0-1 μM). Cells were harvested after 24, 48, and 72 hours and counted under a light microscope after trypan blue exclusion (0.4% trypan blue, Life Technologies, USA) according to instructions of manufacturer. Each treatment was performed in triplicate wells per experiment.

2.5. ROS Level. The production of intracellular ROS was measured using a 6-corboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescent probe (Invitrogen, USA). The DCFH-DA passively enters the cell where it reacts with ROS to form the highly fluorescent compound dichlorofluorescein (DCF). In brief, cells were plated in 6-well plates and allowed to attach overnight. After treatment with the indicated concentrations of MTX for 24 h, the cells were washed 2 times with DMEM without FBS and then loaded with 10 μM DCFHDA at 37°C, 5% CO_2 for 20 min. Cells were washed another three times by PBS and then observed under a fluorescene microscope. ROS was also measured as previously described [19]. The cells were lysed using 1% SDS and sonicated. After 5 min centrifuge (14,000 rpm), supernatant was aliquoted (100 mL) into 96-well plate and measured by Victor 3 microplate reader. Signal intensity was normalized to protein concentration.

2.6. Quantitative RT-PCR. Total RNA was prepared using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA (2 μg) was used to synthesize the first strand of cDNA. Quantitative RT-PCR (qRT-PCR) was performed in a 96-well Bio-Rad CFX96 real time PCR system (Bio-Rad Inc., Hercules, CA) and SYBR Premix Ex Taq kit (Perfect Real Time) (Takara Bio Inc., Shiga, Japan). β-Actin was applied as the inner control. Results are presented as $2^{-\Delta\Delta CT}$ values, defined as the threshold PCR cycle number at which an amplified product is first detected. The $2^{-\Delta\Delta CT}$ was determined as the mean of the triplicate CT values for target gene minus the mean of the triplicate CT values for β-actin. The primers used were P53, forward 5′-CACGCCTGTAATCCCAGCTACTC-3′ and reverse 5′-GCAATGGCACAATCTCGGCTCACT-3′; NOXA, forward 5′-CTTGGAAACGGAGTGGAA-3′ and reverse 5′-CGCCCAGTTAATCACAGGT-3′; XIAF, forward 5′-GCCTGCAAGAAACGAAACTC-3′ and reverse 5′-CTGGCCTCATGGCCTTAT-3′; β-actin, forward 5′-CCCAGCCATGTACGTTGCTA-3′ and reverse 5′-AGGGCATACCCCTCGTAGATG-3′.

2.7. Western Blotting Analysis. Cell lysates were prepared with lysis buffer. The lysates were cleared by centrifugation and total protein concentration was measured using bicinchoninic acid assay kit (Bio-Rad Laboratories). The protein samples were separated on SDS-PAGE at 10–15% and transferred to a polyvinylidene difluoride membrane (Immobilon-P transfer membrane; Millipore) with a Bio-Rad Trans-Blot semidry transfer cell apparatus. The membranes were blocked by 5% nonfat milk in Tris-buffered saline Tween-20 (TBST, pH 7.6) for 90 min at room temperature.

Primary antibodies were diluted in 5% BSA of TBST and incubated for 2 h at room temperature or overnight at 4°C. After washing 3 times with TBST, the membranes were incubated with anti-rabbit, anti-mouse, or anti-goat (1/2,000; R&D Systems) secondary antibodies coupled to horseradish peroxidase for 1 h at room temperature. The membranes were washed 3 times with TBST; then the bands were detected using electrochemiluminescence (ECL) (GE Healthcare UK Ltd.).

2.8. Statistical Analysis. Data were expressed as means ± SD. All experiments were independently repeated at least three times. The statistical significance of differences was determined by Student's two-tailed t-test. $P < 0.05$ was considered statistically significant. Asterisks indicate the level of significance. The data were analyzed using SPSS 17.0.

3. Results

3.1. ERRα Overexpression Blocks MTX-Induced Osteosarcoma Cell Death. To investigate the potential function of ERRα in osteosarcoma (OS) progression, we analyzed ERRα gene expression in Gene Expression Omnibus (GEO) datasets. We found that ERRα expression was significantly upregulated in the metastatic osteosarcomas compared with nonmetastatic tumors in GSE21257 dataset (Figure 1(a)). Metastatic osteosarcomas that are resistant to conventional chemotherapy are the major cause of death. To explore the potential role of ERRα in chemotherapeutic resistance, we first established U2OS stable sublines that overexpress ERRα (U2OS-ERRα number 1 and U2OS-ERRα number 2) (Figure 1(b)). We then examined the effect of ERRα overexpression on the sensitivities of U2OS cells to chemotherapeutic agent MTX. Results in Figures 1(c) and 1(d) demonstrated that overexpression of ERRα strongly protected the cells from MTX-induced growth inhibition. By trypan blue exclusion assay, we found that the elevated ERRα can efficiently block MTX-induced cell death (Figure 1(e)). As shown in Figure 1(f), MTX at a low concentration (0.125 μM) effectively induced apoptosis in the parental U2OS cells, as detected by cleaved PARR and cleaved caspase 7. However, in U2OS-ERRα cells, MTX even at 8-fold higher concentration (1 μM) did not efficiently cause cell apoptosis. These results suggested that ERRα conferred resistance to MTX in human osteosarcoma cells through inhibition of apoptosis.

3.2. MTX Induces Cell Death by Increasing ROS Production in Osteosarcoma Cells. A number of studies indicated that reactive oxygen species (ROS) are involved in a variety of different cellular processes ranging from apoptosis and necrosis to cell proliferation and carcinogenesis [20, 21]. To determine whether ROS is involved in MTX-induced cell death, we stained the U2OS cells with CM2-DCFHDA staining and found that MTX treatment significantly increased ROS production in U2OS cells while N-acetylcysteine (NAC), an antioxidant compound, effectively blocked its induction of ROS (Figures 2(a) and 2(b)). Furthermore, simultaneous treatment with NAC almost completely protected the cells from MTX-induced cell death (Figure 2(c)). Therefore, ROS plays a critical role in MTX-induced cell death.

3.3. ERRα Confers MTX Resistance by Modulating ROS Production in Osteosarcoma Cells. We and others demonstrated previously that ERRα modulates ROS production in cancer cells [12, 22, 23]. We next examined whether ERRα-mediated MTX resistance was through control of ROS. Results shown in Figures 3(a) and 3(b) demonstrated that overexpression of ERRα could efficiently block MTX-induced ROS generation. To further demonstrate that ERRα protected cells from MTX-induced cell death by downregulating ROS production, we treated U2OS parental and U2OS-ERRα cells with hydrogen peroxide (H_2O_2), a highly potent form of ROS. We found that ERRα overexpression also strikingly protected H_2O_2-induced cell growth inhibition even at 50 mM of H_2O_2 (Figure 3(c)). These results collectively suggested that ERRα confers MTX resistance by suppression of ROS production in osteosarcoma cells.

3.4. P53-Mediated Apoptosis Pathway Is Involved in ERRα Mediated MTX Resistance. It is well established that tumor suppressor P53 and its target genes are involved in ROS-induced apoptosis in different cancer cells [24, 25]. We thus examined the effect of ERRα overexpression and MTX on p53 protein level and the role of ROS in this process. Using Western blotting analysis, we found that MTX dose-dependently induced p53 at the protein level and that ERRα overexpression blocked this induction (Figure 4(a)). Consistent with the notion that MTX induction of P53 involves ROS, the antioxidant compound NAC effectively eliminated MTX-induced p53 accumulation (Figure 4(b)). Moreover, P53 induction by H_2O_2 could also be suppressed by ERRα (Figure 4(c)). Importantly ERRα overexpression also blocked MTX-induced NOXA and XAF1 expression which are p53 direct target genes for promoting cell apoptosis (Figures 4(d) and 4(e)). These results collectively indicated that ERRα overexpression suppressed MTX-induced p53 activity through modulating ROS production.

4. Discussion

Osteosarcoma (OS) is the most common tumor of bone. Currently, the use of neoadjuvant chemotherapy combined with surgical resection is the mainstay therapy in OS [3]. The overall survival for osteosarcoma patients has been increased to over 70%. However, the 5-year survival of patients with OS metastasis still remains about 20–30%. Drug resistance is still a significant clinical problem in effective therapy of osteosarcoma. In this study, we showed that overexpressing

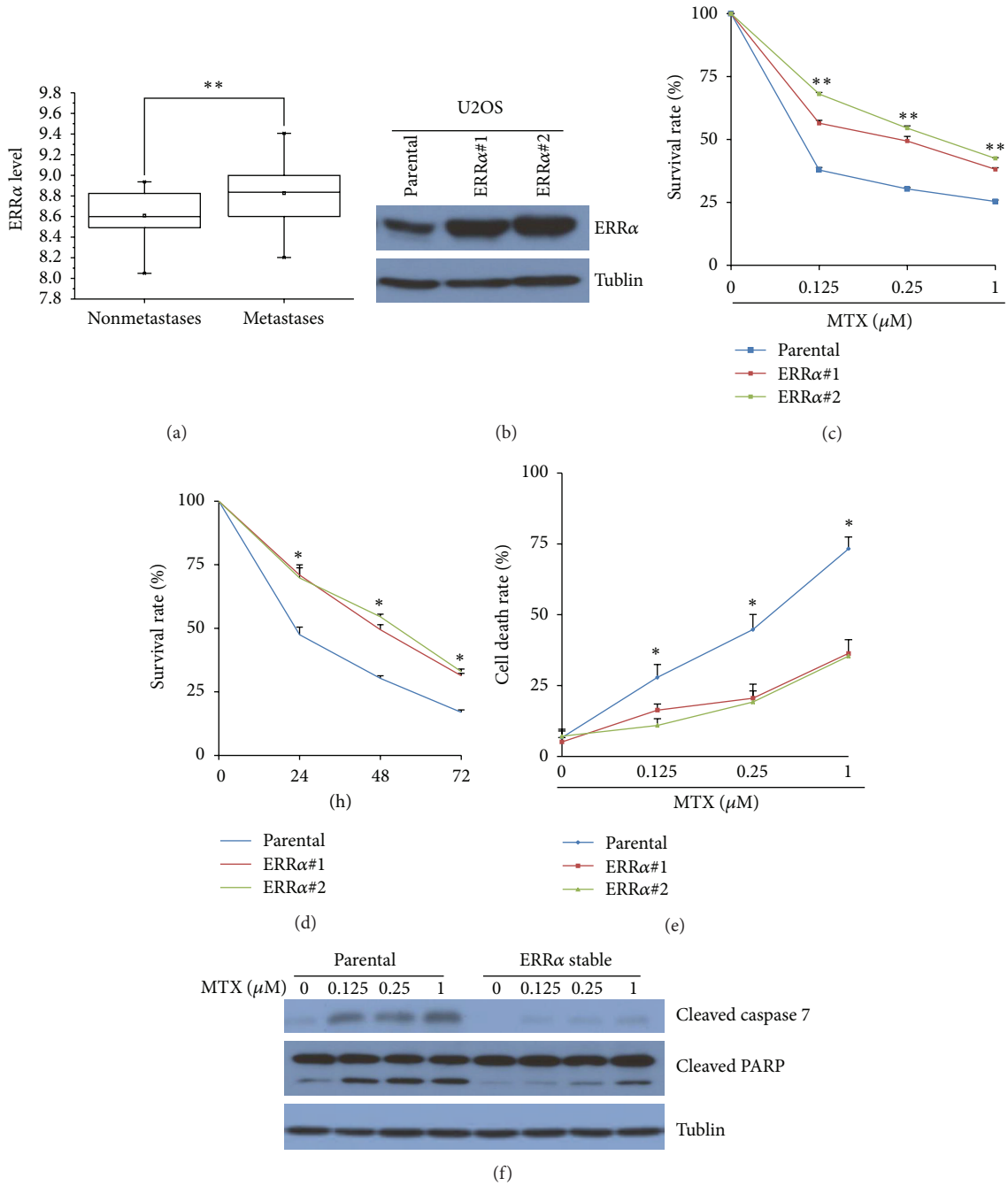

Figure 1: Enhanced ERRα increases resistance to MTX-induced cell death. (a) The expression of the ERRα gene was evaluated from nonmetastasis ($n = 19$) and metastasis ($n = 34$) osteosarcoma in the GEO dataset GSE21257. (b) Western blotting of ERRα gene in U2OS parental and U2OS ERRα overexpression stable cells (U2OS-ERRα). (c) U2OS parental and U2OS-ERRα (numbers 1 and 2) were treated with MTX (0-1 μM) for 48 hours and then survival rate was analyzed by counting cell numbers. (d) U2OS parental and U2OS-ERRα cells (numbers 1 and 2) were treated with 0.25 μM MTX and were collected at 24 hours, 48 hours, and 72 hours. The survival rate was analyzed by counting cell numbers. (e) U2OS parental and U2OS-ERRα cells (numbers 1 and 2) were treated with MTX (0-1 μM) for 72 hours; dead cells were assessed by the trypan blue exclusion assay. (f) U2OS parental and U2OS-ERRα cells (numbers 1 and 2) were treated with MTX (0-1 μM) for 48 hours and then cleaved caspase 7 and cleaved PARP were detected by Western blotting. Data are expressed as mean ± SD ($n = 3$) and analyzed using Student's t-test. Asterisks indicate significant differences: $^{*}P < 0.05$, $^{**}P < 0.01$.

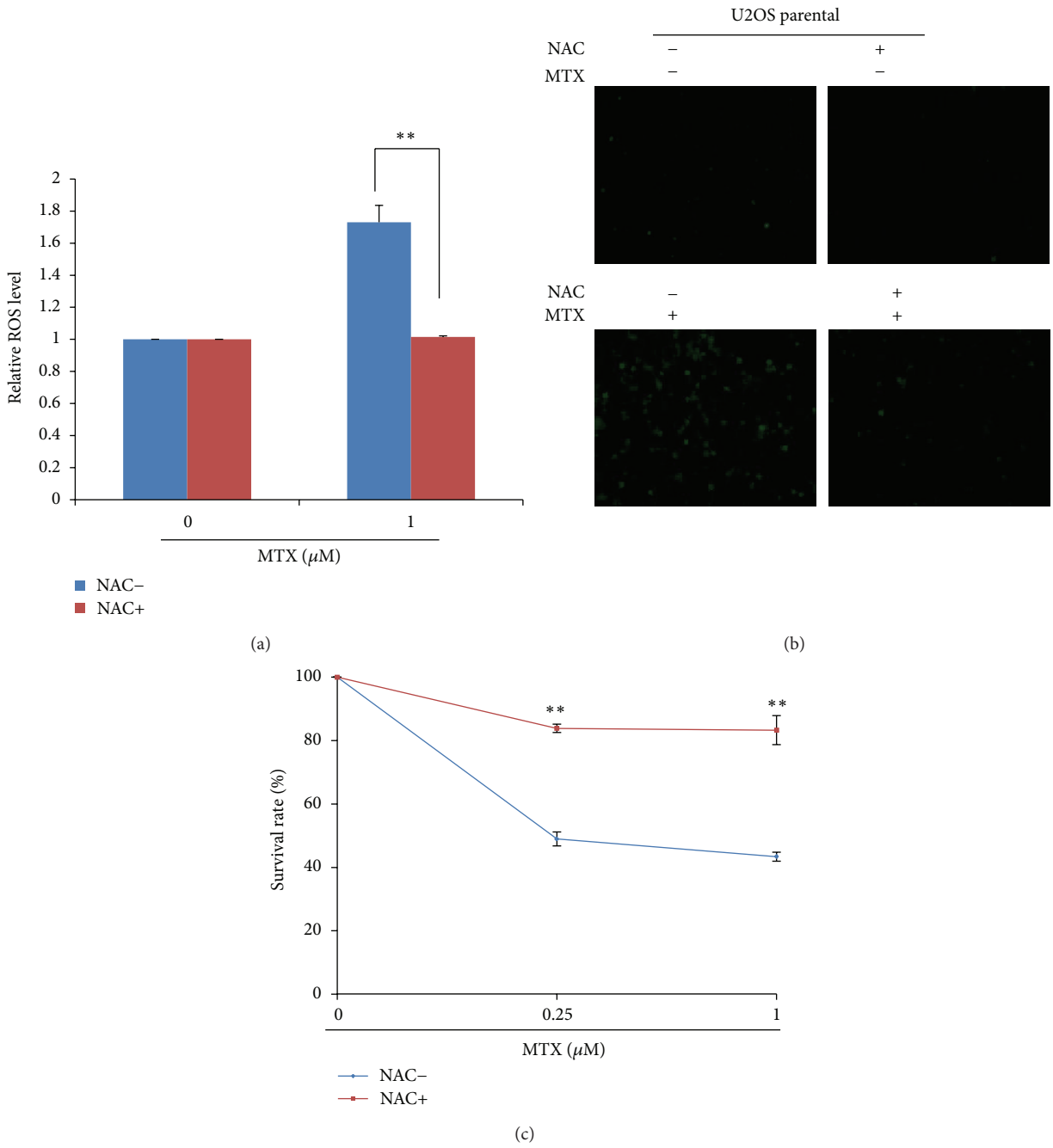

FIGURE 2: MTX induces cell death by increasing ROS production in osteosarcoma cells. ((a)-(b)) U2OS cells were treated with 1 μM MTX for 24 hours; ROS production was analyzed by DCFH-DA staining using microreader and fluorescent microscope. (c) U2OS cells were pretreated with 25 mM NAC for 2 hours followed by treatment with 1 μM MTX for 24 hours. ROS production was analyzed by DCFH-DA staining using microreader and fluorescent microscope. Data are expressed as mean ± SD ($n = 3$) and analyzed using Student's t-test. Asterisks indicate significant differences: $^{*}P < 0.05$, $^{**}P < 0.01$.

ERRα in human osteosarcoma cells promotes the survival of OS cells through inhibiting MTX-induced apoptosis. In addition, our results indicated that ERRα overexpression confers osteosarcoma resistance to MTX via attenuation of reactive oxygen species production and p53-mediated apoptosis pathway in osteosarcoma U2OS cells.

The primary function of ERRα is believed to be the regulation of energy metabolism. Additionally, ERRα at least has been shown to play a role in the regulation of bone formation [26, 27]. ERRα is expressed throughout all developmental stages from early progenitors to bone forming osteoblasts [26]. It may play a functional role in osteoblast differentiation and bone formation by regulating the expression of osteocalcin (OC), bone sialoprotein (BSP), Runx2, and alkaline phosphatase (ALP) [28, 29]. We and others have identified that osteopontin (OPN) and osteocalcin (OC) are ERRα

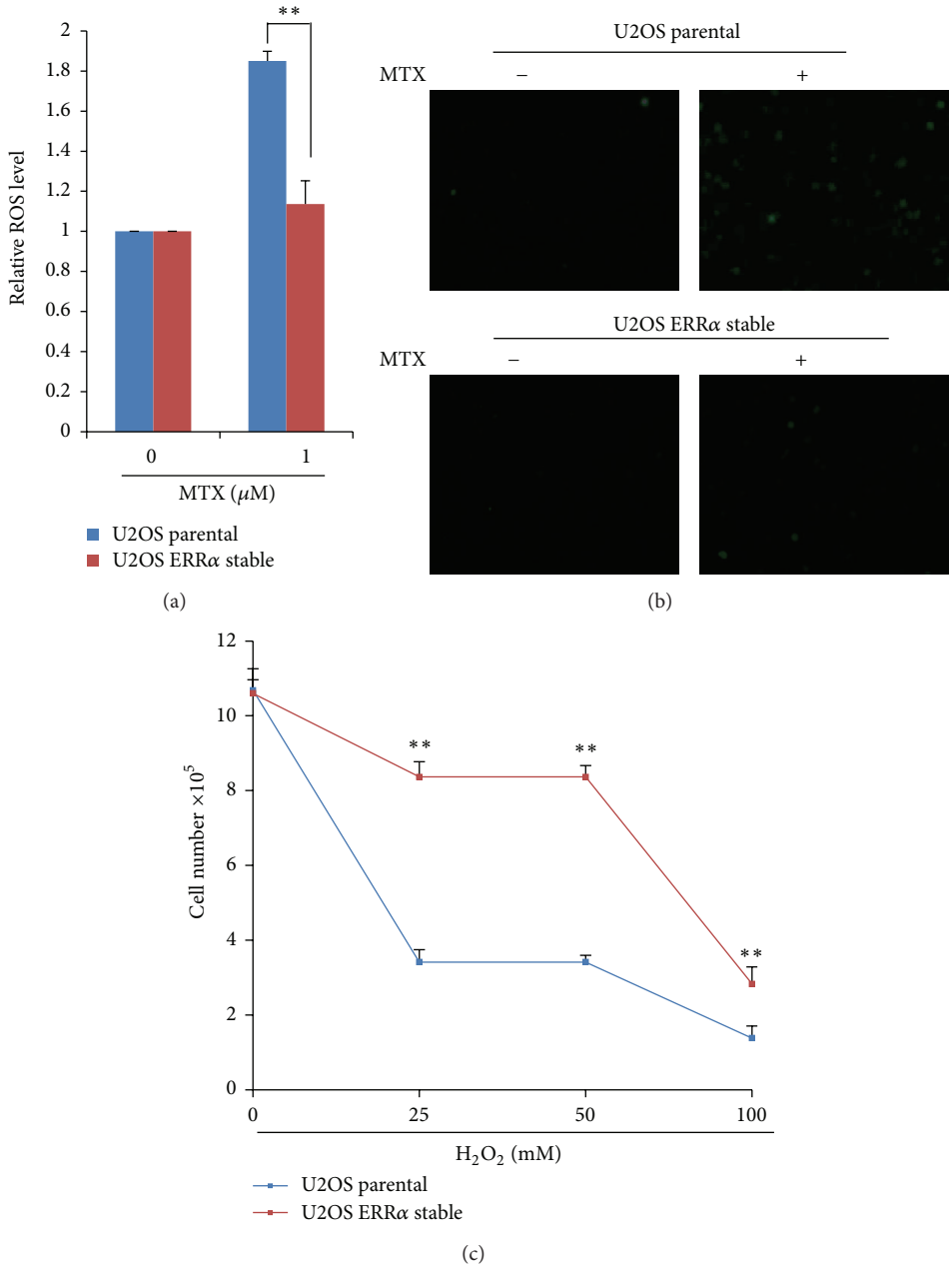

FIGURE 3: ERRα confers MTX resistance by modulating ROS production in osteosarcoma cells. ((a)-(b)) U2OS parental and U2OS-ERRα cells (numbers 1 and 2) were treated with 1 μM MTX for 24 hours and then ROS production was analyzed by DCFH-DA staining using microreader and fluorescent microscope. (c) U2OS parental and U2OS-ERRα cells (numbers 1 and 2) were treated with different concentrations of H_2O_2 (0–100 mM) for 24 hours; cell numbers were counted. Data are expressed as mean ± SD ($n = 3$) and analyzed using Student's t-test. Asterisks indicate significant differences: $^{*}P < 0.05$, $^{**}P < 0.01$.

direct target genes in bone [30, 31]. Interestingly, studies also showed that OPN and OC promote cancer progression in different cancer types [32, 33]. Although it is well established that OPN and OC play important roles in bone formation, their roles in osteosarcoma remain unknown. To understand the role of ERRα and its target genes in osteosarcoma progression, further investigations, including using animal xenograft models, are needed to be done to demonstrate the importance of ERRα in advanced and chemotherapy resistance osteosarcoma. In current research, we demonstrated for the first time that the expression of ERRα is correlated with osteosarcoma progression and that ERRα overexpression can mediate MTX resistance in osteosarcoma cells. Our data collectively suggested that ERRα may be a novel target for combating chemotherapy resistance and advanced osteosarcoma.

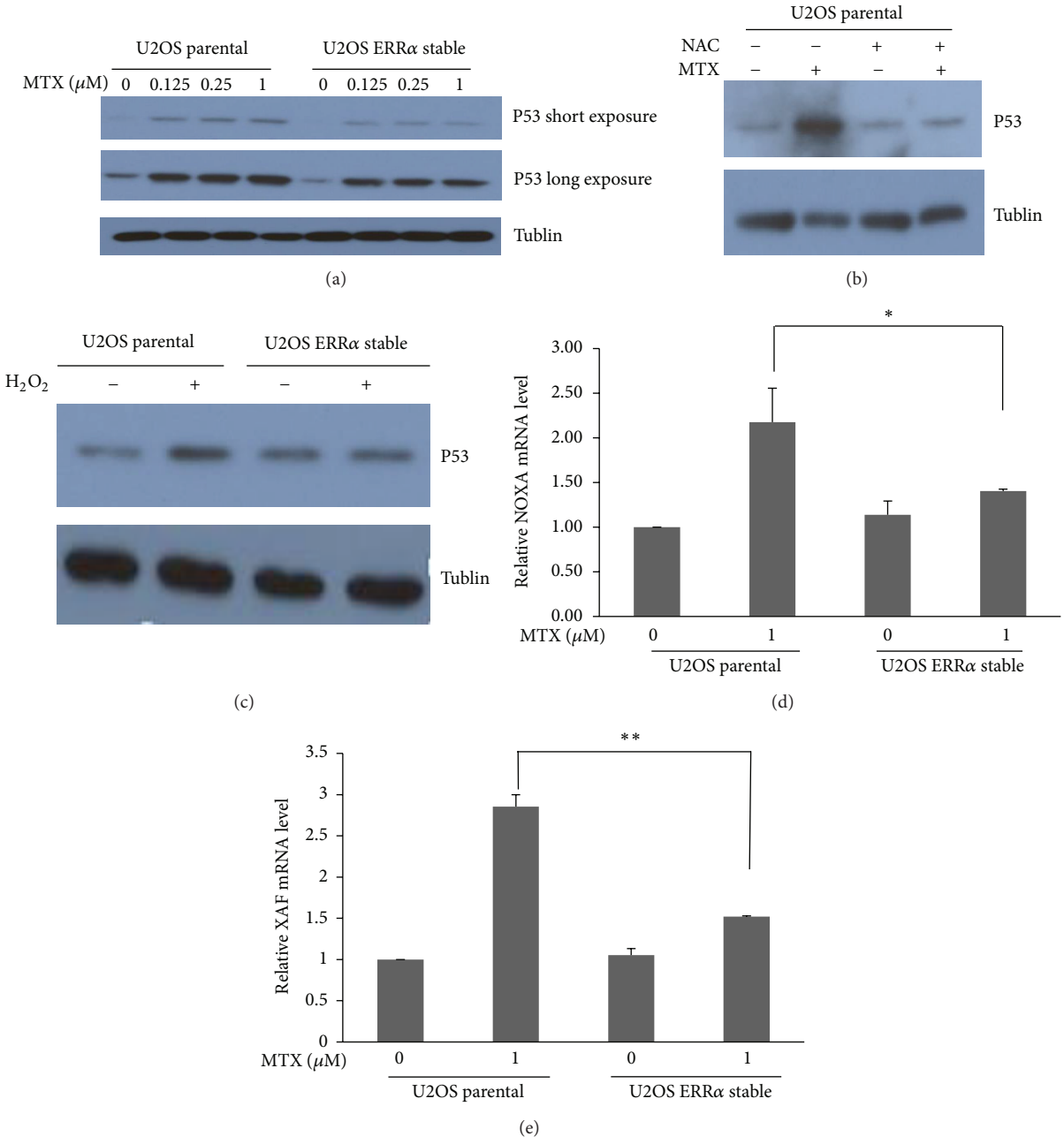

Figure 4: P53 apoptosis pathway is involved in ERRα mediated MTX resistance. (a) U2OS parental and U2OS-ERRα cells (numbers 1 and 2) were treated with MTX (0-1 μM) for 48 hours; P53 protein level was assayed by western blotting. (b) U2OS parental cells were pretreated with 25 mM NAC for 2 hours followed by treatment with 1 μM MTX for 48 hours; P53 protein level was assayed by western blotting. ((c)–(e)) U2OS parental and U2OS-ERRα cells (numbers 1 and 2) were treated with 1 μM MTX for 48 hours, the expression of NOXA, XAF, and P53 was detected by QPCR. (f) U2OS parental and U2OS-ERRα cells (numbers 1 and 2) were treated with 25 mM H_2O_2 for 24 hours; P53 protein level was assayed by western blotting. Data are expressed as mean ± SD ($n = 3$) and analyzed using Student's t-test. Asterisks indicate significant differences: $^{*}P < 0.05$, $^{**}P < 0.01$.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Peng Chen and Haibin Wang contributed equally to this paper.

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Review Article

Current and Emerging Biomarkers of Cell Death in Human Disease

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Cell death is a critical biological process, serving many important functions within multicellular organisms. Aberrations in cell death can contribute to the pathology of human diseases. Significant progress made in the research area enormously speeds up our understanding of the biochemical and molecular mechanisms of cell death. According to the distinct morphological and biochemical characteristics, cell death can be triggered by extrinsic or intrinsic apoptosis, regulated necrosis, autophagic cell death, and mitotic catastrophe. Nevertheless, the realization that all of these efforts seek to pursue an effective treatment and cure for the disease has spurred a significant interest in the development of promising biomarkers of cell death to early diagnose disease and accurately predict disease progression and outcome. In this review, we summarize recent knowledge about cell death, survey current and emerging biomarkers of cell death, and discuss the relationship with human diseases.

1. Introduction

Cell death is a fundamental biological process which has been mediated via intracellular program of biological systems [1–3]. Growing evidence has provided an expanding view for the existence of various types of cell death. Nonetheless, with different criteria, cell death can be classified into different subroutines and different subroutines of cell death own a distinct molecular mechanism and morphological characters and perform different roles in regulating the fate of cells [4]. And then along with progress and substantial insights into the biochemical and molecular mechanism exploration of cell death, its classification from the initial morphology has now been transformed to the biochemical characteristics. A functional classification suggested by the Nomenclature Committee on Cell Death (NCCD) based on the biochemical characteristics, including extrinsic as well as intrinsic apoptosis, regulated necrosis, autophagic cell death, and mitotic catastrophe, has been widely accepted [5].

In natural state cell death plays an important role during the development, maintenance of tissue homeostasis, and elimination of damaged cells [1]. One of the typical examples is that once a cell infected by virus has DNA damage or cell cycle disturbed, cell death will eliminate this cell to ensure the normal life activities of organism [1, 6]. On the contrary, excessive or defective cell death contributes to a broad spectrum of human pathologies; low-rate cell death can result in cancer formation and autoimmune disease [7–9], while high-rate cell death can result in neurodegenerative disease, immunodeficiency, and muscle atrophy [10–13]. Insights into the molecular mechanisms involved in cell death will likely have important implications and offer the opportunity to target this process for therapeutic purposes. However, the rational treatment design and selection are often precluded due to the lack of adequate biomarkers for stratifying patient subgroups. Therefore, central to current research and clinical efforts is the need for finding cell death biomarkers for early detection, diagnosis, and prognosis that can provide more

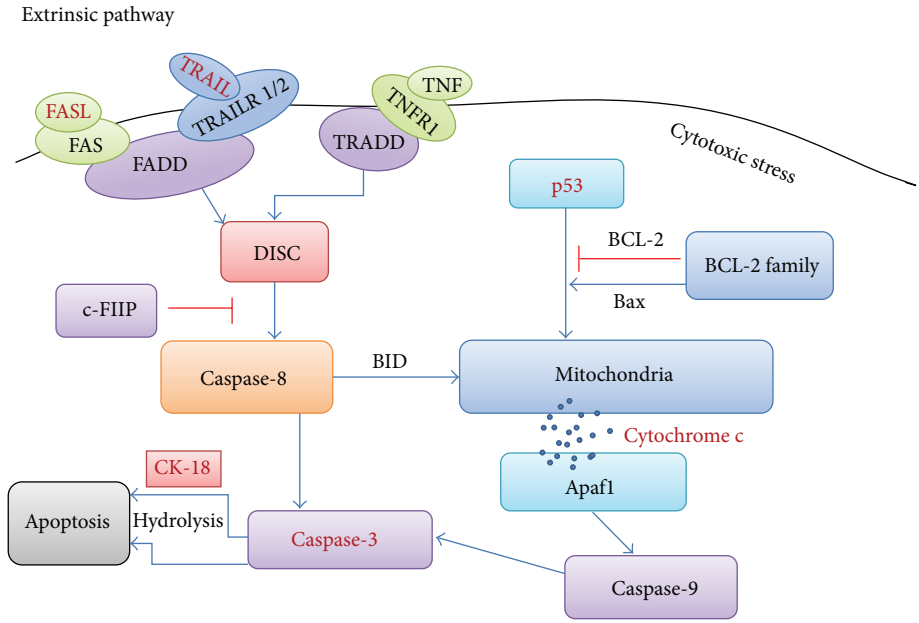

Figure 1: A schematic diagram of apoptosis.

accurate personalized management [14]. In this review, we summarize recent literatures on cell death biomarkers and discuss the relationship with human diseases.

2. Extrinsic as well as Intrinsic Apoptosis

Apoptosis, being a highly complex and sophisticated process, involves a series of complex biochemical events leading to a spatiotemporal sequence of morphological changes, such as nuclear condensation and fragmentation, as well as plasma membrane blebbing [15]. Characteristic biochemical events of cells undergoing apoptosis include activation of effectors caspases (caspase-3, caspase-6, and caspase-7), mitochondrial outer membrane permeabilization (MOMP), and activation of catabolic hydrolases [16]. Apoptosis can occur via extrinsic and intrinsic pathways, which are initiated either by extracellular death receptors, such as FAS, TNF-α, and TRAIL, or by intracellular stimuli, such as DNA damage, hypoxia, and nutrient deprivation [12] (Figure 1). Of note, the signaling cascades triggering intrinsic apoptosis are highly heterogeneous, whose triggering can proceed in a caspase-dependent or caspase-independent manner [17]. Moreover, there is accumulating evidence that cross-talk exists between extrinsic and intrinsic pathways [18].

Extrinsic apoptosis is always initiated by the activation of Fas cell surface death receptor (FAS) or TNF-related apoptosis-inducing ligand (TRAIL) which can recruit the adaptor molecules, Fas-associating protein with death domain (FADD), while it also can be stimulated by TNFR1 which can recruit TNFR1-associated death domain (TRADD). The activated FADD or TRADD leads to the formation and activation of DISC activating caspase-8. As an inhibitor, c-FIIP inactivates caspase-8 to suppress the apoptosis. The activated caspase-8 promotes the activation of caspase-3, which in turn induces the characteristics of apoptosis. Intrinsic apoptosis is triggered by cytotoxic stress resulting in the activation of p53, which promotes mitochondrial cytochrome c release into the cytosol. This process can be regulated by BCL-2 family and also triggered by BID which stimulated by extrinsic pathway. The dissociative cytochrome c binds with Apaf1 from the apoptosome to activate caspase-9. Then caspase-3 will be activated by caspase-9 finally resulting in apoptosis. In addition, caspase-3 promotes the apoptosis through hydrolyzing Ck-18 during the final stage.

3. Autophagic Cell Death

Autophagy is an essential and conserved catabolic process, which is initiated by the nucleation of isolation membrane [19] (Figure 2). This is followed by the expansion of this membrane to form the autophagosome and fuse with the lysosome to degrade cellular components [20]. Autophagic cell death is mediated by autophagy and autophagy-related proteins and that is characterized by mTOR suppression as well as Atg activation and reaction [21]. When subjected to a variety of stress stimuli, such as energy depletion or nutrient deprivation [22], autophagic cell death can be initiated by the enhanced autophagic flux, which can also be prevented by the suppression of autophagy by chemicals and/or genetic means, such as agents targeting VPS34 or RNAi targeting essential autophagic modulators, such as ATG5 or Beclin 1 [23, 24]. It should be noted that the precise molecular mechanisms regulating autophagic cell death remain to be determined [25].

The initiation of autophagy is triggered during the starvation environment which leads to the activation of AMP-activated protein kinase (AMPK) and inactivation of the rapamycin complex 1 (mTORC1). Both of these two mechanisms can promote the formation and activation of ULK1

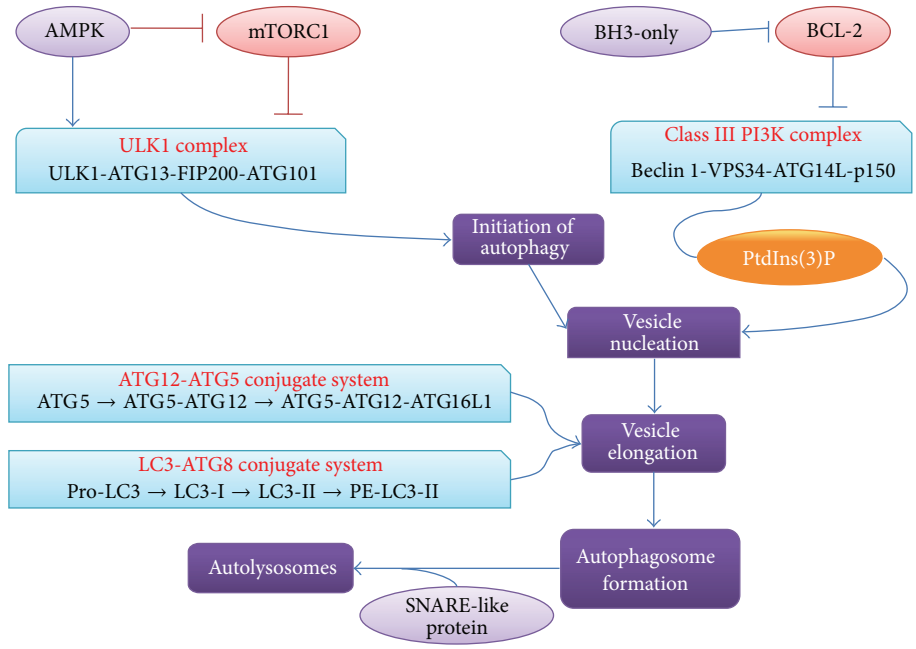

Figure 2: A schematic diagram of autophagic cell death.

complex which consists of ULK1, ATG13, FIP200, and ATG101. Vesicle nucleation mainly involves the activation of autophagy-specific class III PI3 K complex to form phosphatidylinositol-3-phosphate (PtdIns(3)P). Class III PI3 K complex can be inactivated by BCL-2, while BCL-2 homology 3- (BH3-) only proteins can induce autophagy by competitively disrupting the interaction between Beclin 1 and BCL-2. Vesicle elongation process involves two ubiquitin-like conjugation systems: ATG12-ATG5 conjugate system and LC3-ATG8 conjugate system. Once the vesicle was completed, an autophagosome formed. Then the fusion between autophagosome and lysosome is mediated by several SNARE-like proteins and forms autolysosomes.

4. Regulated Necrosis

Regulated necrosis, being a genetically controlled process, can occur in a highly regulated manner [26] and is characterized by a series of morphological changes, including cytoplasmic granulation, as well as organelle and/or cellular swelling [26]. Meanwhile, regulated necrosis is accompanied by some biochemical events, including caspase inhibition, NADPH oxidase activation, and NET release [27, 28]. Regulated necrosis can be triggered in response to a variety of physicochemical insults, including alkylating DNA damage, excitotoxins, and the ligation of death receptors [5]. Clearly, substantial advances in the characterization of the molecular mechanisms have rapidly increased our understanding of regulated necrosis. With regard to its dependence on specific signaling pathways, regulated necrosis can be further divided into different types characterized by (but not limited to) necroptosis, mitochondrial permeability transition- (MPT-) dependent regulated necrosis, and parthanatos [5, 29] (Figure 3). Of note, they are interconnected and overlapping with each other at the molecular level that impinges on common mechanisms, such as redox metabolism and bioenergetics, to result in the similar morphology [26].

Regulated necrosis at least can be divided into three different pathways, including necroptosis, MPT-dependent regular necrosis, and parthanatos. In the necroptosis pathway, the activation of TNF-alpha/FASL binds to their receptors TNFR1/FAS to activates the RIPK1 and RIPK3 which in turn phosphorylate the mixed lineage kinase domain-like (MLKL). The activated MLKL promotes the activation of plasma membrane permeabilization (PMP) and then triggers the necrosis. During this pathway, caspase-8, FLIPL, and FADD act as inhibitors of regular necrosis to suppress the activation of RIPK3. In the MPT-dependent regular necrosis pathway, transition pore complex (PTPC) plays a key role in mitochondrial permeability transition which leads to the abrupt increase of ROS and Ca^{2+} in the cytoplasm, resulting in the regular necrosis. In the parthanatos pathway, poly-ADP-ribose polymerase 1 (PARP1) starts to repair the damaged DNA, leading to the decrease of ATP and the hyperactivation of apoptosis-inducing factor (AIF) promoting the regular necrosis.

5. Mitotic Catastrophe

Mitotic catastrophe acts as an oncosuppressive mechanism that can occur either during or after mitosis to precede cell apoptosis, necrosis, or senescence [30]. It is characterized by unscheduled activation of cyclin B1-CDK1, TP53, or TP73, caspase-2 activation, and mitotic arrest [30–33]. Several processes have been shown to be dispensable for mitotic catastrophe that can be initiated in response to a series of triggers, including perturbation of the mitotic apparatus and chromosome segregation early in mitosis [30, 34]. During the

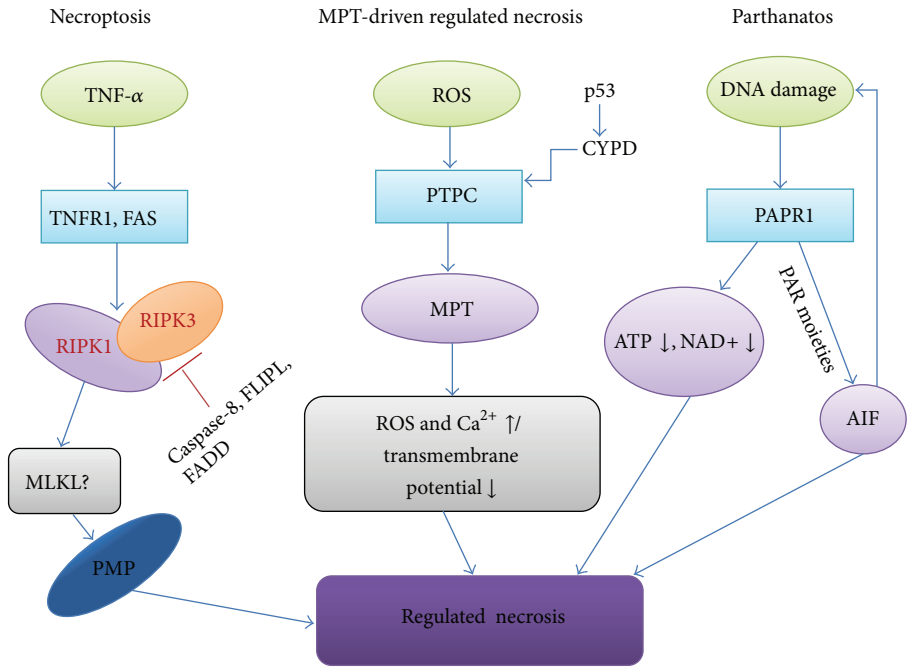

Figure 3: A schematic diagram of regulated necrosis.

past few years, although progress has unraveled a myriad of pathways that can induce mitotic catastrophe, it is still poorly understood [30, 35].

6. Current and Emerging Biomarkers of Cell Death

Currently, much attention has been given to cell death and focused on developing biomarkers, but only a few of cell death-related genes have been identified as molecular biomarkers. Most frequently described are the death receptors and their ligands, caspases, cytokeratin-18, p53, and others (Table 1). More recently, breakthroughs have identified a number of noncoding RNAs as biomarkers such as microRNAs and lncRNAs that are present in the execution of cell death [36, 37].

6.1. Current Biomarker. Death receptors are membrane-bound protein complexes that can activate an intracellular signaling cascade by binding specific ligands and play a central role in apoptosis [12, 38]. Death receptors belong to the TNFR (tumor necrosis factor receptor) superfamily whose members typically include Fas (also known as CD95, APO-1, and TNFRSF6), TNFR1 (also known as CD120a, p55, and p60), TRAILR1 (also known as DR4, CD261, and APO-2), and TRAILR2 (also known as DR5, KILLER, and CD262) [39]. These death receptors contain a cytoplasmic region of ~80 residues termed the death domain (DD) which provides the capacity for protein-protein interactions with other molecules [40]. Here, the most extensively studied death ligands are type II transmembrane proteins, including FasL (for Fas receptor), TNF (for TNFR1 receptor), and TRAIL (for TRAIL receptor) [41, 42]. After proteolytic cleavage of the membrane-anchored ligand, these ligands are released from the plasma membrane and enable them to bind to death receptors and trigger their activation [40]. Upon contacting with their corresponding ligands, these receptors are triggered, leading to the recruitment of a different set of adaptor molecules to the death domain and subsequent activation of the signaling cascade, where the major signals transmitted by death receptors such as Fas, TNFR1, TRAILR1, and TRAILR2 result in an apoptotic response mediated by intracellular caspases [43–48]. The above-mentioned associations give our hints and strategy guides for the death receptors and their ligands as potential biomarkers. In support of this notion, some of them have been shown to be utilized as biomarkers. For example, soluble Fas ligand is identified as a biomarker of thyroid cancer recurrence and may be useful for risk-adapted surveillance strategies in thyroid cancer patients [49]. Costagliola et al. demonstrated that TNF-alpha in tears can be used as a biomarker to assess the degree of diabetic retinopathy [50].

Caspases are a family of endoproteases that play an important role in maintaining homeostasis through regulating cell death [51]. According to their mechanism of action, caspases can be classified into two major types: one is initiator caspases, including caspase-2, caspase-8, caspase-9, and caspase-10, and the other is effector caspases, including caspase-3, caspase-6, and caspase-7. Furthermore, initiators can be subdivided into caspases that participate in either the extrinsic (caspase-8 and caspase-10) or the intrinsic (caspase-2 and caspase-9) pathway [52]. As we know, the prodomain of different caspases is different, allowing them to interact with other different molecules that regulate their activities [51]. For example, caspase-1, caspase-2, caspase-4, caspase-5, caspase-9, caspase-12, and caspase-13 contain a caspase recruitment domain (CARD), whereas caspase-8 and

Table 1: Cell death biomarkers in human diseases.

Official symbol	Official full name	Clinical relevance	Function	Pathway	References
CASP3	Caspase-3, apoptosis-related cysteine peptidase	A potential new biomarker for myocardial injury and cardiovascular disease	Caspase-3 is responsible for chromatin condensation and DNA fragmentation	Apoptosis	[96]
TP53	Tumor protein p53	Implications for the regulation and execution of apoptosis in colorectal cancer and other cancers.	TP53 activation is capable of inducing apoptosis by intrinsic pathway.	Apoptosis	[97]
KRT18	Keratin 18	A biomarker of liver damage and apoptosis in chronic hepatitis C	CK18-Gly(−) involves the inactivation of Akt1 and protein kinase Cθ	Apoptosis	[98–100]
FAS	Fas cell surface death receptor	Granulomatous disease	Fas can increase the antigen-specific CD8(+) T-cell responses during viral infection	Apoptosis	[101, 102]
TRAIL	Tumor necrosis factor (ligand) superfamily, member 10	Inducing the autoimmune inflammation in SLE	TRAIL directly induces apoptosis through an extrinsic pathway, which involes the activation of caspases.	Apoptosis	[103]
MAP1LC3A	Microtubule-associated protein 1 light chain 3 alpha	Neurodegenerative and neuromuscular diseases, tumorigenesis, and bacterial and viral infections	LC3-II functions in phagophore expansion and also in cargo recognition	Autophagy	[104, 105]
BECN1	Beclin 1, autophagy related	Human breast cancers and ovarian cancers	BECN1 is part of a Class III PI3K complex that participates in autophagosome formation, mediating the localization of other autophagy proteins.	Autophagy	[106]
RIPK1	Receptor (TNFRSF)-interacting serine-threonine kinase 1	Involving retinal disorders including retinitis pigmentosa and retinal detachment	RIPK1 and RIPK3 association forms a necrosis-inducing complex, initiates cell-death signals (programmed necrosis).	Necrosis	[107–109]
RIPK3	Receptor-interacting serine-threonine kinase 3	Atherosclerotic lesions and the pathogenesis of inflammatory bowel	RIPK3 interacts with, and phosphorylates RIPK1 and MLKL to form a necrosis-inducing complex, then triggering necrosis.	Necrosis	[110–112]

caspase-10 have a death effector domain (DED) [53–55]. With complexing capacity of different molecules, caspases can be activated in different ways via granzyme B, death receptors, or apoptosome [56, 57]. For example, granzyme B, which can be released by cytotoxic T lymphocytes and NK cells, is able to activate caspase-3 and caspase-7 [58]. Fas, TRAIL, TNF, and other receptors can activate caspase-8 and caspase-10 [59, 60]. Again, apoptosome that is regulated by cytochrome c and the BCL-2 family can activate caspase-9 [61]. Initiator caspases promoting the caspase cascade reaction result in the activation of effector caspases which is achieved by cleavage of their inactive proforms and then trigger the apoptotic process [51]. There has been extensive effort to identify caspase as biomarkers, the most typical example being caspase-3 [12, 62–64]. For example, Simpson et al. indicated that the active mutant caspase-3 induced by doxycycline to drive synchronous apoptosis plays key roles in human colorectal cancer cells [62]. Singh et al. speculated that caspase-3 may be a potential new biomarker for myocardial injury and cardiovascular disease [63].

Cytokeratin-18 (CK-18) and other cytokeratins constitute the type I intermediate filaments of the cytoskeleton, which is present in epithelial cells [65]. CK-18, one of the most prominent substrates for lethal caspase activation, can be cleaved by caspases, primarily not only by caspase-9, but also by caspase-3 and caspase-7, and the subsequent release of CK-18 fragments into the extracellular space occurs during cell death [66]. Notably, there are several molecular forms of CK-18 released from dying cells that can be distinguished conveniently [67]. For example, apoptosis will lead to the release of caspase-cleaved CK-18 fragments, and necrosis will lead to release of uncleaved CK-18 [68]. Multiple studies have demonstrated that CK-18 and CK-18 fragments can be released from cells into blood [66, 67, 69, 70], suggesting the potential use of CK-18 fragments or CK-18 as noninvasive biomarkers of human diseases. Vos et al. measured plasma CK-18 levels in normal weight children and obese children with and without nonalcoholic fatty liver disease (NAFLD) and found that its level is elevated in children with suspected NAFLD and was proposed as a diagnostic biomarker of NAFLD [69]. Feldstein et al. found that serum CK-18 fragment can be used as a useful biomarker for nonalcoholic steatohepatitis (NASH) in children with fatty liver disease [71]. In the review of usefulness of cytokeratin- (CK-) 18 fragments, the authors state that the caspase-cleaved fragment of cytokeratin-18 is a marker of chronic liver disease [72].

However, it should be noted that some issues with regard to the stability, reliability, and beneficial clinical utility of CK-18 and CK-18 fragments still need to be verified and answered.

In addition, DNA damage can also stimulate the transactivation of genes encoding proapoptotic proteins and trigger the apoptotic process in a p53-dependent manner [73]. The p53 is an important proapoptotic factor that is inactivated in a normal cell by its negative regulators [74]. MDM2 is the main negative regulator of p53 activity and stability [75]. A wide range of cellular stress stimuli, including DNA damage, hypoxia, and oncogene activation, can cause dissociation of the p53 from MDM2 complex [76, 77]. Once activated, p53 will induce apoptotic cell death by activating a series of positive regulators of apoptosis such as DR-5 and Bax [78, 79]. There is mounting evidence that p53 and MDM2 genes are used as biomarkers of cell death [80, 81]. Patil et al. reported using p53 as a prognostic biomarker of breast cancer [82]. Li et al. demonstrated that p53 immunohistochemical expression may serve as prognostic marker for the survival of oral squamous cell carcinoma (OSCC) patients receiving surgery [83]. Barone et al. indicated that targeting the interaction between p53 and its negative regulator MDM2 represents a new major therapeutic approach in poor prognosis of paediatric malignancies without p53 mutations [80].

6.2. Emerging Biomarker. Noncoding RNAs, including microRNAs and long noncoding RNAs (lncRNAs), are key regulatory molecules involved in multiple cellular processes. MicroRNAs are about 22 nt small noncoding RNA molecules, which function in transcriptional and posttranscriptional regulation of gene expression via mRNA cleavage or translational arrest [84]. It is established that they play an important role in cell death related pathway including autophagy and apoptosis [85–87]. Comparing to microRNA, lncRNAs are over 200 nt noncoding RNAs, which are emerging as new players in gene regulation as posttranscriptional regulators of splicing or as molecular decoys for microRNA [88]. Besides, some other mechanisms have been proposed to explain its mediated gene expression by lncRNA [89]. Although many lncRNAs have been identified, of all lncRNAs only few have been well characterized. Currently, emerging evidence suggests that microRNAs and lncRNAs may serve as diagnostic or prognostic biomarkers of human diseases [90]. For example, microRNA-21 (miR-21) is shown to be involved in apoptosis as well as inflammatory and fibrotic signaling pathways in acute kidney injury, which is now considered a novel biomarker aiding diagnosis and treatment of acute kidney injury [91]. MiR-497 is a potential prognostic marker in human cervical cancer and functions as a tumor suppressor by inducing caspase-3-dependent apoptosis to decrease cell growth [92]. MiR-181a functions as an oncogene by negatively regulating PRKCD, a promoter of apoptosis, to induce chemoresistance in cervical squamous cell carcinoma cells, and may provide a biomarker for predicting chemosensitivity to cisplatin in patients with cervical squamous cancer [93]. Recent demonstration that lncRNA silencing in preclinical models leads to cancer cell death and/or metastasis prevention, suggesting that they can be investigated as novel biomarkers, has triggered increasing interest [37]. An lncRNA has recently been found to play an important role in the growth and tumorigenesis of human gastric cancer and may be a potential biomarker for gastric cancer [94]. Weber et al. indicated that lncRNA MALAT1 might be applicable as a blood-based complementary biomarker for the diagnosis of non-small cell lung cancer [95].

7. Conclusions

In the past few years, accumulated knowledge continues to improve our understanding of the biological and biochemical processes during cell death. We have witnessed tremendous advances in the discovery and identification of novel cell death biomarkers for early detection, diagnosis, and prognosis of human diseases, with some biomarkers now in clinical utility. However, there are still debate and challenges regarding the cell death related biomarkers, including availability, stability, and accuracy of biomarkers. As far as a good biomarker is concerned, it should be present in peripheral body fluid and/or tissue such as blood, urine, and saliva. Second, it should be easy to detect, preferably in a quantifiable manner. Third, it should associate as specifically as possible with diseases. To achieve the goal, we need to find a clear path for biomarker translation from discovery to clinical practice.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Kongning Li, Deng Wu, and Xi Chen contributed equally to this work.

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Research Article

Predicting the Types of J-Proteins Using Clustered Amino Acids

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J-proteins are molecular chaperones and present in a wide variety of organisms from prokaryote to eukaryote. Based on their domain organizations, J-proteins can be classified into 4 types, that is, Type I, Type II, Type III, and Type IV. Different types of J-proteins play distinct roles in influencing cancer properties and cell death. Thus, reliably annotating the types of J-proteins is essential to better understand their molecular functions. In the present work, a support vector machine based method was developed to identify the types of J-proteins using the tripeptide composition of reduced amino acid alphabet. In the jackknife cross-validation, the maximum overall accuracy of 94% was achieved on a stringent benchmark dataset. We also analyzed the amino acid compositions by using analysis of variance and found the distinct distributions of amino acids in each family of the J-proteins. To enhance the value of the practical applications of the proposed model, an online web server was developed and can be freely accessed.

1. Introduction

J-protein, also known as Hsp40 (heat shock protein 40 kD), is a molecular chaperone protein and is found ubiquitously in both prokaryotes and eukaryotes [1, 2]. J-proteins represent a large family of molecular chaperones and have cooperative functions with Hsp70. Most of the J-proteins contain a "J" domain through which they can interact with and stimulate Hsp70. Based on the structural differences, J-proteins can be classified into four types, that is, Type I, Type II, Type III, and Type IV J-proteins. Type I J-proteins contain an N-terminal J-domain that is separated from the rest of the proteins by a linker "G/F" region (glycine/phenylalanine region) [3, 4]. Distal to G/F region is the zinc-binding cysteine-rich sequence named as "Zinc-finger domain" which distinguishes Type I proteins from other types of J-proteins [4], and Zinc-finger domain is followed by the C-terminal domain [1, 2]. Type II proteins possess all the domains in Type I except the zinc-finger domain [3]. Type III J-proteins contain a C-terminal J-domain but lack both G/F and zinc-finger domains [3]. Type IV, also known as the J-like protein [5], is a group of recently identified proteins that lacks histidine, proline, and aspartate signature motifs in their sequences [4].

By binding Hsp70 and Hsp90, J-proteins play important roles in chaperone cycle regulation and control many physiological functions [4], such as assisting the folding of nascent and damaged proteins, translocation of polypeptides across cellular membranes, and degradation of misfolded proteins [6]. Studies carried out in the past decade have also shown the regulatory roles of J-proteins in cell death. In association with Hsp70, J-proteins not only involve in the folding of caspase-activated DNase which is responsible for the apoptosis-induced DNA fragmentation [7] but also protect the macrophages from nitric-oxide-mediated apoptosis [8]. Gotoh and his colleagues have demonstrated the role of J-protein in the inhibition of Bax translocation to the mitochondria to prevent nitric-oxide-induced cell apoptosis [9]. Kurisu et al. found that MDG1/ERdj4, a member of

Table 1: Breakdown of the benchmark dataset used in current study.

Total number	Subfamily	Number
1245	Type I J-protein	63
	Type II J-protein	53
	Type III J-protein	1107
	Type IV J-protein	22

the human J-protein family, can interact with GRP78/BiP and protect against the cell death induced by endoplasmic reticulum stress in human [10]. The regulation of cell death by J-protein was also reported in plant. Liu and Whitham found that the overexpression of J-protein stimulated the hypersensitive response (HR)-like cell death in soybean [11]. Cancer progressions are also reported to be closely related to J-proteins, but different types of J-proteins play distinct roles [12, 13]. Type I J-protein is tumour promoting, while Type II J-protein acts as tumour suppressors [13]. Therefore, reliably annotating the types of J-proteins is of major importance in order to clarify their distinct biological functions in cell death. However, to the best of our knowledge, there is no computational method for predicting the types of J-proteins.

Keeping these in mind, in the present work, we proposed a model to predict the four functional types of J-proteins based on reduced amino acid alphabet compositions. According to a recent review [14], the rest of the papers are organized as follows: (i) construct a valid benchmark dataset to train and test the predictor; (ii) formulate the samples with an effective mathematical expression that can truly reflect their intrinsic correlation with the target to be predicted; (iii) select a powerful machine learning method to operate the prediction; (iv) perform cross-validation tests to objectively evaluate the anticipated accuracy of the predictor; (v) provide a web server for the prediction method.

2. Materials and Methods

2.1. Dataset. The sequences of J-protein were taken from the HSPIR database at http://pdslab.biochem.iisc.ernet.in/hspir/, which currently contains 3,901 J-protein sequences [15]. To reduce homologous bias, J-proteins that have ≥40% pairwise sequence identity to each other were removed by using the CD-HIT program [16]. By doing so, we obtained a benchmark dataset containing 1,245 J-proteins that were classified into four types: 63 Type I J-proteins, 53 Type II J-proteins, 1,107 Type III J-proteins, and 22 Type IV J-proteins (Table 1). The benchmark dataset can be freely downloaded from http://lin.uestc.edu.cn/server/iJPred/data.

2.2. Reduced Amino Acid Alphabet. Based on the physiochemical properties, the 20 native amino acids can be clustered into a smaller number of representative residues called reduced amino acid alphabet (RAAA) [17–19]. Compared with the traditional amino acid composition, RAAA not only simplifies the complexity of protein system but also improves the ability in finding structurally conserved regions and structural similarity of entire proteins.

Table 2: Scheme for reduced amino acid alphabet based on protein blocks method.

Cluster profiles	Protein blocks method
CP(13)	G-**IV**-**FYW**-A-L-M-E-**QRK**-P-**ND**-**HS**-T-C
CP(11)	G-**IV**-**FYW**-A-**LM**-**EQRK**-P-**ND**-**HS**-T-C
CP(9)	G-**IV**-**FYW**-**ALM**-**EQRK**-P-**ND**-**HS**-**TC**
CP(8)	G-**IV**-**FYW**-**ALM**-**EQRK**-P-**ND**-**HSTC**
CP(5)	G-**IVFYW**-**ALMEQRK**-P-**NDHSTC**

Recently, a structural alphabet called protein blocks (PBs) was proposed by de Brevern et al. [20, 21] and has been widely used in computational proteomics as indicated in a review [22]. To aid the design of mutations, Etchebest and his colleagues defined a novel type of RAAA based on PBs [23], where the 20 native amino acids can form five different cluster profiles, that is, CP(13), CP(11), CP(9), CP(8), and CP(5) as shown in Table 2. Ever since it was proposed, RAAA has been widely used for protein family classifications [24–27].

Hence, in the present study, the J-proteins were encoded using the RAAA as formulated by the discrete feature vector **P**:

$$\mathbf{P} = [f_1 \quad f_2 \quad \cdots \quad f_i \quad \cdots \quad f_D]^{\mathbf{T}}, \tag{1}$$

where **T** is the transposing operator and f_i is the occurrence frequency of the ith n-peptide RAAA and defined as

$$f_i = \frac{N_i}{L - n + 1}, \tag{2}$$

where N_i is the number of the ith$n$-peptide ($n = 1$, 2, or 3) RAAA in a J-protein with length of L. For the different cluster profiles (Table 2) and different values of n, the vector dimension (D) in (1) will be different. The corresponding dimensions of reduced amino acid ($n = 1$) composition, reduced dipeptide ($n = 2$) composition, and reduced tripeptide ($n = 3$) composition were listed in Table 3.

2.3. Support Vector Machine (SVM). SVM is a powerful and popular method for pattern recognition that has been widely used in the realm of bioinformatics [28–41]. The basic idea of SVM is to transform the data into a high dimensional feature space and then determine the optimal separating hyperplane using a kernel function. To handle a multiclass problem, "one-versus-one (OVO)" and "one-versus-rest (OVR)" methods are generally applied to extend the traditional SVM. For a brief formulation of SVM and how it works, see the papers [28, 29].

In the current study, the LIBSVM 2.84 package [42] was used as an implementation of SVM, which can be downloaded from http://www.csie.ntu.edu.tw/~cjlin/libsvm/. The OVO method was employed for making predictions using the popular radial basis function (RBF). The regularization parameter C and the kernel width parameter γ were determined via an optimization procedure using a grid search approach using the fivefold cross-validation. In grid research, the search spaces for parameter C and γ range from 2^{15} to 2^{-5} and from 2^{-5} to 2^{-15} with the steps of 2^{-1} and 2, respectively.

Table 3: Feature vector dimension of n-peptide composition with different cluster profiles.

n-peptide	Cluster profiles				
	CP(13)	CP(11)	CP(9)	CP(8)	CP(5)
$n = 1$	13	11	9	8	5
$n = 2$	169	121	81	64	25
$n = 3$	2197	1331	729	512	125

2.4. Performance Evaluation. The performance of the method was measured in terms of sensitivity (Sn), specificity (Sp), Matthew's correlation coefficient (MCC), and overall accuracy (OA) defined as follows:

$$\begin{aligned} \mathrm{Sn}(i) &= \frac{\mathrm{TP}(i)}{\mathrm{TP}(i) + \mathrm{FN}(i)}, \\ \mathrm{Sp}(i) &= \frac{\mathrm{TN}(i)}{\mathrm{TN}(i) + \mathrm{FP}(i)}, \\ \mathrm{MCC}(i) &= \frac{\mathrm{TP}(i) \times \mathrm{TN}(i) - \mathrm{FP}(i) \times \mathrm{FN}(i)}{\sqrt{[\mathrm{TP}(i) + \mathrm{FP}(i)]\,[\mathrm{TP}(i) + \mathrm{FN}(i)]\,[\mathrm{TN}(i) + \mathrm{FP}(i)]\,[\mathrm{TN}(i) + \mathrm{FN}(i)]}}, \\ \mathrm{OA} &= \frac{1}{N}\sum_{i=1}^{M} \mathrm{TP}(i), \end{aligned} \tag{3}$$

where TP(i), TN(i), FP(i), and FN(i) represent true positive, true negative, false positive, and false negative of family i; M is the number of subsets and equals to 4, while N is the number of the total J-proteins in benchmark dataset.

3. Results and Discussion

3.1. Cross-Validation. Three cross-validation methods, namely, subsampling (or K-fold cross-validation) test, independent dataset test, and jackknife test, are often used to evaluate the quality of a predictor [43]. Among the three methods, the jackknife test is deemed the least arbitrary and most objective as elucidated in [44] and hence has been widely recognized and increasingly adopted by investigators to examine the quality of various predictors [31, 34, 45–50]. Accordingly, the jackknife test was used to examine the performance of the model proposed in the current study. In the jackknife test, each sequence in the training dataset is in turn singled out as an independent test sample and all the rule parameters are calculated without including the one being identified.

The jackknife results obtained by the proposed model on the benchmark dataset based on the five different cluster profiles of the tripeptide (i.e., $n = 3$) case were listed in Table 4. As it can be seen from Table 4, the best success rate of 94.06% was achieved when the predictions were based on CP(8) with a dimension of 512. For comparison, the results of the amino acid (i.e., $n = 1$) and dipeptide (i.e., $n = 2$) cases were also calculated and listed in Table 5, from which we can see that none of them has higher success rates than the case of $n = 3$.

In our previous study [27], the six HSP families were successfully classified by using the dipeptide of RAAA. But for the classification of the J-protein subfamilies in the present work, the best predictive result was obtained by using the tripeptide of RAAA. Hsps belong to the same family share more sequence identity than that of different families [5]; hence we need more suitable parameters to encode the protein sequences as used in the current study.

3.2. Comparison with Other Methods. Since there is no published work to predict the types of J-proteins, we could not provide the comparison analysis with existing results to confirm that our presented model is superior to other methods. However, for the purpose of comparison, we compared the results of the present model with that of Random Forest and Naïve Bayes using the same optimal features (the reduced tripeptide compositions based on CP(8)). The results of jackknife test on the benchmark dataset for Random Forest and Naïve Bayes are listed in Table 6. It is shown that the accuracy of SVM is higher than that of Random Forest and Naïve Bayes.

3.3. Amino Acids Composition Analysis. To provide an overall view, the frequencies of the 20 naive amino acids were compared among the four types of J-proteins using the analysis of variance (ANOVA), and the average amino acid frequency of one type of J-protein with that of another type was further explored and compared using the Fisher's least significant difference (LSD) test. The result is given in Figure 1, where the green boxes indicate that the frequency differences among different types of J-proteins are not significant, while blue and red boxes indicate that the frequency differences are

Table 4: Results obtained in identifying J-protein functional types with tripeptide case ($n = 3$).

Subfamily	Metrics	Feature dimension of $n = 3$ for each cluster profile				
		CP(13)	CP(11)	CP(9)	CP(8)	CP(5)
		2197	1331	729	**512**	125
Type I J-protein	Sn	63.49%	74.60%	77.78%	**74.60%**	60.31%
	Sp	99.56%	98.94%	99.11%	**98.76%**	98.93%
	MCC	0.74	0.76	0.79	**0.75**	0.66
Type II J-protein	Sn	37.73%	45.28%	39.62%	**49.06%**	24.53%
	Sp	100%	99.31%	99.39%	**99.05%**	99.56%
	MCC	0.60	0.57	0.53	**0.57**	0.41
Type III J-protein	Sn	99.81%	98.82%	99.09%	**98.56%**	99.19%
	Sp	44.44%	58.78%	55.72%	**62.02%**	40.00%
	MCC	0.63	0.68	0.67	**0.69**	0.56
Type IV J-protein	Sn	0	27.27%	13.64%	**31.81%**	4.54%
	Sp	100.00%	100.00%	100.00%	**100.00%**	100.00%
	MCC	0	0.52	0.37	**0.56**	0.21
OA		93.57%	94.06%	93.98%	**94.06%**	92.36%

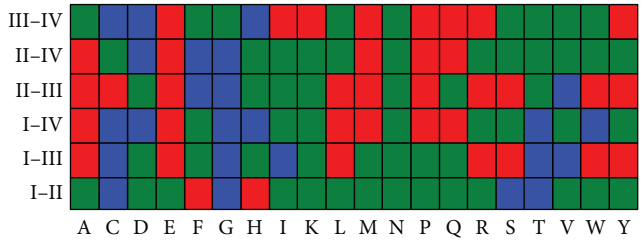

Figure 1: Statistical results to show the divergent distributions of the 20 amino acids among the four (I, II, III, and IV) types of J-proteins. The green boxes indicate that the frequency differences among different types of J-proteins are not significant. The blue boxes indicate that the amino acid is significantly enriched ($P < 0.05$; LSD test) in one type of J-proteins compared with its counterpart. Taking W as an example, the blue box with the coordinate (W, I–IV) indicates that W is enriched in Type I J-proteins compared with Type IV J-proteins. The red boxes indicate that the amino acid is lacking in one type of J-proteins but significantly enriched ($P < 0.05$; LSD-test) in its counterpart. Also taking W as the example, the two red boxes with the coordinates (W, I–III) and (W, II-III) indicate that W is lacking in both Type I and Type II J-proteins compared with Type III J-proteins, respectively.

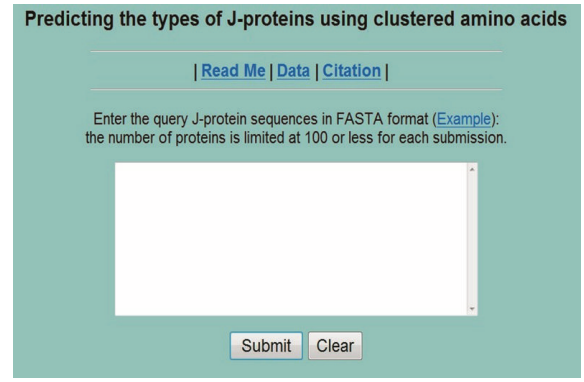

Figure 2: A semiscreenshot to show the top page of the web server. It is available at http://lin.uestc.edu.cn/server/Jpred.

significant ($P < 0.05$; LSD test) among different types of J-proteins (see Figure 1 for more details).

We found that, except Asn (N), the frequencies of all the other 19 amino acids are significantly different among the four types of J-proteins. Compared with other three types, Type I J-proteins are enriched in Cys (C), Gly (G), and Thr (T), Type II J-proteins are enriched in Phe (F), Type III J-proteins are enriched in Ala (A) and Leu (L), while Type IV-J proteins are enriched in Met (M), Gln (Q), Glu (E), and Pro (P) but lack Asp (D) and His (H). The lack of D and H residues in Type IV-J proteins leads to their inability to stimulate ATP hydrolysis [5]. Moreover, according to the binomial distribution [51], we also found the overpresented tripeptides in each family and listed them in Supporting Table S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2014/935719, where the over-presented tripeptides with their confidence levels are provided. These results indicate that the distinct distributions of amino acids in the four types of J-proteins may account for their distinct functions in biological processes.

3.4. Web Server Guide. To enhance the value of the practical applications of the proposed model and for the convenience of the vast majority of experimental scientists, an online predictor was developed. The step-by-step guide on how to use it is provided as follows.

(1) Open the web server at http://lin.uestc.edu.cn/server/Jpred and you will see the top page as shown in Figure 2. Click on the *Read Me* button to see a brief introduction about the predictor and the caveat when

Table 5: Results obtained in identifying functional types with (a) single amino acid case ($n = 1$) and (b) dipeptide case ($n = 2$).

(a) For the single amino acid case ($n = 1$)

Subfamily	Metrics	Feature dimension of $n = 1$ for each cluster profile					
		CP(20)	CP(13)	CP(11)	CP(9)	CP(8)	CP(5)
		20	13	11	9	8	5
Type I J-protein	Sn	71.42%	65.08%	68.25%	52.38%	50.79%	22.22%
	Sp	98.58%	98.66%	98.66%	98.93%	98.48%	98.03%
	MCC	0.71	0.67	0.69	0.60	0.56	0.26
Type II J-protein	Sn	33.96%	30.19%	33.96%	16.98%	16.98%	15.09%
	Sp	99.82%	99.21%	99.12%	99.47%	99.56%	99.09%
	MCC	0.54	0.42	0.45	0.30	0.31	0.23
Type III J-protein	Sn	98.74%	98.28%	98.10%	99.09%	98.73%	98.19%
	Sp	48.12%	42.86%	45.52%	32.31%	31.54%	17.46%
	MCC	59.71%	0.53	0.54	0.48	0.45	0.26
Type IV J-protein	Sn	4.54%	0	0	0	0	0
	Sp	99.91%	100.00%	100.00%	100.00%	100.00%	100.00%
	MCC	0.15	0.53	0	0	0	0
OA		92.93%	91.97%	92.13%	91.48%	91.08%	89.08%

(b) For the dipeptide case ($n = 2$)

Subfamily	Metrics	Feature dimension of $n = 2$ for each cluster profile					
		CP(20)	CP(13)	CP(11)	CP(9)	CP(8)	CP(5)
		400	169	121	81	64	25
Type I J-protein	Sn	74.42%	60.31%	73.02%	60.32%	58.73%	49.20%
	Sp	97.58%	98.59%	98.76%	97.71%	98.32%	97.79%
	MCC	0.75	0.63	0.73	0.58	0.60	0.5
Type II J-protein	Sn	39.76%	45.23%	39.62%	39.62%	35.84%	28.30%
	Sp	94.31%	99.29%	99.48%	99.03%	98.60%	97.99%
	MCC	0.57	0.57	0.54	0.49	0.42	0.31
Type III J-protein	Sn	98.88%	98.10%	98.82%	97.74%	98.01%	97.31%
	Sp	46.37%	50.74%	51.14%	50.79%	48.80%	40.34%
	MCC	60.08%	0.59	0.62	0.57	0.56	0.46
Type IV J-protein	Sn	13.16%	27.27%	0	22.73%	25.00%	9.09%
	Sp	99.91%	99.91%	100.00%	100.00%	100.00%	99.91%
	MCC	0.13	0.48	0	0.47	0.47	0.24
OA		91.47%	92.93%	93.25%	91.97%	92.04%	91.16%

Table 6: Comparative result of SVM with other methods for J-protein types classification.

Subfamily	SVM			Random Forest			Naïve Bayes		
	Sn	SP	MCC	Sn	SP	MCC	Sn	SP	MCC
Type I J-protein	74.60%	98.76%	0.75	14.29%	99.55%	0.29	74.60%	92.17%	0.47
Type II J-protein	49.06%	99.05%	0.57	13.33%	99.82%	0.31	54.72%	94.67%	0.39
Type III J-protein	98.56%	62.02%	0.69	99.73%	12.70%	0.31	88.62%	65.83%	0.43
Type IV J-protein	31.81%	100.00%	0.56	4.55%	100.00%	0.21	13.64%	100.00%	0.37
OA		94.06%			89.96%			85.14%	

using it, and click on the *Data* button to download the benchmark datasets used to train and test the predictor. The relevant papers that document the algorithm of the predictor can be found by clicking on the *Citation* button.

(2) Either type or copy/paste the query J-protein sequence into the input box at the center of Figure 2. The input protein sequence should be in the FASTA format that can be seen by clicking on the *Example* button right above the input box.

(3) Click on the *Submit* button to see the predicted result. For example, if you use the four query J-protein sequences in the *Example* window as the input, after clicking the *Submit* button, you will obtain the results: the outcome for the 1st query sample is "*Type I J-protein*;" the outcome for the 2nd query sample is "*Type II J-protein*;" the outcome for the 3rd query sample is "*Type III J-protein*;" the outcome for the 4th query sample is "*Type IV J-protein*."

4. Conclusion

Cell death is a common phenomenon in developmental processes or in normal physiological conditions and is induced by an array of extra- or intracellular stimuli [7]. However, organisms are equipped with their own physiological defense to cope with environmental stress in order to prevent or induce cell death depending upon the severity of the stress [7]. In mammalian cells, the stress response involves the induction of Hsps, such as Hsp70 and Hsp90. By interacting with J-proteins, these Hsps play pivotal roles in cell death regulations. Since J-proteins act as intermediates, the analysis of J-proteins functions is urgent in order to clarify the regulatory roles of Hsps in cell death.

Based on combination of whole-genome analyses and biochemical evidences, a large number of J-proteins have been identified [6]. However, the exact roles for many of the J-proteins are far from being understood [2, 52]. In order to understand its biological functions, it is highly desirable to know which family a given J-protein belongs to.

By encoding the sequences using the reduced amino acid alphabet information, a predictor was developed to identify the four different families of J-proteins in the present work. To enhance the value of the practical applications of the proposed model and for the convenience of the experimental scientists, an online web server was provided and can be freely accessed at http://lin.uestc.edu.cn/server/Jpred. We hope that the present model will be helpful for scientists who focus on J-proteins and will provide novel insights into the research of cell death.

Conflict of Interests

There is no conflict of interests with any financial organization regarding this paper.

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