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Molecular mechanisms of oxaliplatin-induced p53 expression and apoptosis

Graduate School of Chosun University

Department of Bio New Drug Development

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TABLE OF CONTENTS

TABLE OF CONTENTS……...i

LIST OF FIGURES……..iv

LIST OF TABLES...……...……vi

ABBREVIATIONS……vii

ABSTRACT IN KOREAN……..ix

CHAPTER 1: INTRODUCTION.....1

CHAPTER 2: MATERIALS AND METHODS.....9

2.1. Cell culture and drug treatment……..9

2.2. Morphological detection of apoptosis and necrosis by Hoechst 33342 and propidium iodide staining……..9

2.3. SDS-PAGE and Western blot analysis ……10

2.4. RNA interference……..11

2.5. Caspase-8 and -3 activity assay.....13

2.6. *In vitro* protein-lipid binding assay…….....14

2.7. Luciferase activity assay.....14

2.8. Immunofluorescence…….....15

2.9. Subcellular protein fractionation……..16

2.10. Quantitative real-time PCR……16

2.11. Lipid raft and non-raft fractionation and protein precipitation……..17

2.12. Protein qualification…….....18

2.13. Statistical analysis.....18

CHAPTER 3: RESULTS……19

3.1. p53 is necessary for oxaliplatin-induced apoptosis……..19

3.2 Oxaliplatin-induced p53 transcription in a NF-kB-dependent manner……26

3.3. Multiple death receptor signaling controls the NF-kB-dependent p53 expression……29

3.4. Oxaliplatin-induced p53 expression is regulated through multiple death receptor pathway…….....32

3.5. FasL and TRAIL is not involved in oxaliplatin-induced p53 expression …….....36

3.6. Oxaliplatin-induced apoptosis is dependent on multiple death receptors……38

3.7. Apoptosis-inducing proteins are localized at lipid rafts fraction in oxaliplatin-induced apoptosis……..42

3.8. Membrane rafts play a critical role(s) in oxaliplatin-induced apoptosis but not in NF-kB/p53 expression pathway ……..…….…….....46

3.9. p53 facilitates the localization of death receptors into lipid rafts and caspase-8-initiated apoptosis……..……..50

3.10. Oxaliplatin-induced p53 interacts with components of lipid raft…….....55

3.11. p53 enhances the localization of death receptors into lipid rafts and apoptosis……..…..59

CHAPTER 4: DISCUSSION…….…….62

CHAPTER 5: CONCLUSION……69

CHAPTER 6: ABSTRACT IN ENGLISH…….....70

CHAPTER 7: REFERENCES……72

ACKNOWLEDGEMENT…….....84

LIST OF FIGURES

Figure 1: Structure of cisplatin and oxaliplatin…….. 1

Figure 2: Role of p53 in oxaliplatin induces apoptosis …….....22

Figure 3: Oxaliplatin induces p53 expression in wild-type p53-harboring cells……24

Figure 4: Oxaliplatin-induced p53 accumulation is not controlled by proteosomal protein degradation in HepG2 and SK-Hep1 cells... ……..25

Figure 5: Role of NF-kB in oxaliplatin-induced p53 transcription...…….....28

Figure 6: Involvement of multiple death receptors in p53 expression …….....30

Figure 7: Involvement of multiple death receptors in NF-kB activation …….....31

Figure 8: Oxaliplatin-induced p53 expression is regulated through multiple death receptor pathway …….....34

Figure 9: Caspase-8 and pan-caspases are not involved in oxaliplatin-induced p53 expression…….....35

Figure 10: Oxaliplatin-induced p53 expression is not regulated by FasL and TRAIL….....37

Figure 11: Involvement of multiple death receptors in oxaliplatin induces apoptosis ……...40

Figure 12: Oxaliplatin treatment does not increase protein levels of death receptor…….....41

Figure 13: Oxaliplatin induces accumulation of p53 in membranous fraction.....43

Figure 14: Localization of apoptosis inducing proteins in oxaliplatin induces apoptosis ….....44

Figure 15: Oxaliplatin-induced translocation of p53 and DISC fractions into the lipid rafts is disrupted by MBCD.....45

Figure 16: Membrane rafts play a critical role in oxalipaltin-induced apoptosis.....48

Figure 17: Membrane rafts do not affect on oxaliplatin-induced NF-kB/p53 expression pathway……...49

Figure 18: p53 facilitates the localization of death receptors into lipid rafts and caspase-8-initiated apoptosis…….…….....52

Figure 19: Role of p53 in caspase-8 and -3 induces apoptosis…….....54

Figure 20: Interaction of p53 with components of lipid raft …….....……56

Figure 21: Effect of oxaliplatin on the binding of p53 to various lipid components ……..58

Figure 22: p53 enhances the localization of death receptors into lipid rafts and apoptosis…....60

Figure 23: A positive feedback regulation between p53 and death receptor/caspase-8-initiated apoptotic pathway in oxaliplatin-induced apoptosis…….....61

LIST OF TABLES

Table 1: Primers for RNA interference…….....12

ABBREVIATION

BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CHX	Cycloheximide
CtxB	Cholera toxin B
DACH	1, 2-diaminocyclohexane
DED	Death effector domain
DIG	Glycosphingolipid-enriched domain
DISC	Death-inducing signaling complex
DMEM	Dulbecco's modified eagle medium
DR4	Death receptor 4
DR5	Death receptor 5
FADD	Fas-associated death domain
FAS	Fas associate signaling
FBS	Fetal bovine serum
GPI	Glycosylphosphatidylinositol
HCC	Hepatocellular carcinoma
HO	Hoechst
IKB	Inhibitor of kappa B
IKK	Ihibitor of kappa B kinase
MBCD	Methyl beta cyclodextrin
MDM2	Mouse doubles minute 2
MMT	Mismatch repair
MPT	Mitochondrial permeability transition
$Na^{+/}K^{+}$-ATPase	Sodium potassium-adenosine triphosphate

NCI	National cancer institute
NF-kB	Nuclear factor-kappa B
NIK	NF-kB inducing kinase
PBS	Phosphate buffer saline
PI	Propidium iodide
PIP	Phosphoinositide
RHD	Rel homology domain
RIP	Receptor interacting protein
RT-PCR	Real time-polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
siRNA	Small interference ribonucleic acid
SST	Sequence-specific transactivation
TBST	Tris-buffer saline tween 20
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TRAF	Tumor necrosis factor receptor-associate factor
TRAIL	Tumor necrosis factor-related apoptosis inducing ligand
Z-VAD-fmk	Benzyloxycarbonyl-val-ala-asp (ome)-fluoromethylketone

국문초록

Molecular mechanisms of oxaliplatin-induced p53 expression and apoptosis

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바이오 신약개발학과

옥살리플라틴은 플라티늄 구조를 가진 항암제로써, 기존에 널리 사용되던 시스플라틴에 비해 전이암을 비롯한 다양한 조직의 종양에서 한층 증진된 항암효과를 나타내는 것으로 알려져 있다. 본 연구는 옥살리플라틴에 의한 항암 기전을 조사하기 위한 목적으로 진행 되었는데, 옥살리플라틴은 종양 억제 단백질인 p53 의 발현을 증가시키며, p53-의존적으로 암세포의 고사를 유도하였다. 특히 일반적으로 약물 치료에 둔감하게 반응하는 간유래 종양 세포 주에서도 효과적인 세포 사멸 효과를 나타내므로 간유래 종양 세포 주 HepG2 와 SK-Hep1 을 중심으로 옥살리플라틴에 의한 p53 발현 기전과 p53 에 의한 세포 사멸 유도 기전을 조사하였다.

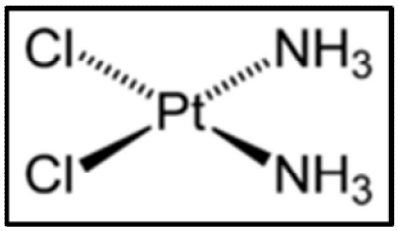
일반적으로 DNA 손상인자에 의한 세포 내 p53 단백질의 축적은 p53 억제 인자인 Mdm2 로부터 분리됨으로써 유비퀴틴화-프로테아좀에 의한 분해 경로로부터의 회피가 주요인으로 알려져 있으나, HepG2 와 SK-Hep1 에서는 mRNA 전사의 증진에 의한 p53 단백질 증가가 확인되었다. p53 의 전사는 세포막에 존재하는 세포 사멸

수용체인 Fas, TRAIL-R1 과 TRAIL-R2 에 의해 조절되었으며, NIK-IKK-IKB-NFkB 경로를 거쳐 조절되었다. 한편 이렇게 옥살리플라틴에 의해 증가된 p53 단백질은 핵 내부뿐 만 아니라 상당량이 세포막에 위치한다는 사실이 확인되었다. p53 의 세포막에서의 역할을 규명하고자 리피드래프트 영역을 분리한 결과 p53 이 세포 사멸 수용체들과 함께 리피드래프트에 위치하며, p53 이 래프트에 위치할 때 DISC 형성이 촉진된다는 결과를 얻었다. 또한 세포 면역 염색법에서도 p53 의 래프트 이동이 관찰되었다. p53 의 리피드 래프트로의 이동은 대장암 세포주인 HCT116 에서도 동일하게 확인되었다. 이때 옥살리플라틴과 함께 래프트 형성을 억제하는 물질인 MBCD 를 처리하면, p53 과 DISC 의 래프트로의 이동이 억제됨과 동시에 세포고사가 저해되지만 세포 사멸 수용체를 통한 NFkB 경로와 p53 발현 증가는 억제되지 않으므로, p53 에 의한 세포 사멸 수용체의 활성화와 DISC 형성 촉진을 통한 caspase-8 의 활성화 및 세포 고사 유도 경로는 래프트-의존적으로 조절되지만 세포 사멸 수용체를 통한 NFkB/p53 발현 증가는 래프트에 비의존적으로 조절되는 것으로 유추된다. 그리고 siRNA 를 이용하여 p53 의 발현을 저하시키자, 옥살리플라틴에 의한 caspase-8 과 caspase-3 의 활성은 저하되는 반면, NFkB 의 전사 활성은 오히려 증가 되었다. 또한 HepG2 세포에서 얻어진 단백질을 다양한 지질 성분과 반응시킨 결과 옥살리플라틴이 처리된 경우 p53 이 리피드 래프트 구성 지질인 3-sulfogalactosylceramide, phosphatidic acid 와 다양한 phosphoatidyl inositol phosphate 들과 결합한다는 사실을 확인 하였다. 위의 결과들을 종합할 때, 옥살리플라틴은 세포사멸 수용체를 통하여 p53 의 발현과 caspase-8 의 활성화 경로를 모두 활성화 시킴으로써 효과적인 세포 고사를 유도하며, 이때 p53 의 래프트 존재 유무에 따라 세포 사멸 수용체의 역할이 조절 될 수 있음을 시사한다.

CHAPTER 1: INTRODUCTION

Oxaliplatin is a platinum based chemotherapeutic drug in the same family of cisplatin and carboplatin (Fig. 1). Compared to cisplatin the two amine groups are replaced by diaminocyclohexane (DACH) for improve antitumor activity. The chlorine ligands are replaced by the oxalate bidentate derived from oxalic acid in order to improve water solubility [1].

a. Cisplatin



b. Oxaliplatin

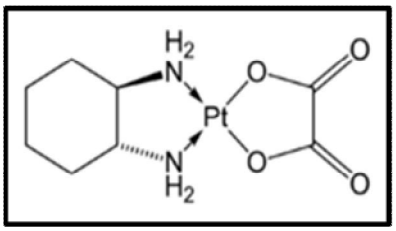


Figure 1. Structure of cisplatin and oxaliplatin (Nature Reviews/ Drug Discovery 3, 11-12 January 2004).

Oxaliplatin was initially launched in France in 1996 and, more recently, in the United States in August 2002. Oxaliplatin, an agent with a DACH carrier ligand, has shown *in vitro* and *in vivo* efficacy against many tumor cell lines, including some that are resistant to cisplatin and carboplatin. The retention of the bulky DACH ring by activated oxaliplatin is thought to result in the formation of platinum-DNA adducts, which appear to be more effective in blocking DNA replication and are more cytotoxic than adducts formed from cisplatin. Studies by the National Cancer Institute (NCI) have suggested that oxaliplatin has a spectrum of activity different from that of either cisplatin or carboplatin. It has different molecular targets and/or mechanisms of resistance [2]. Oxaliplatin has been demonstrated to differ in some

mechanisms associated with the development of cisplatin resistance. Compared with cisplatin-conditioned cells, deficiencies in mismatch repair (MMR) and increases in replicative bypass, which appear to contribute to cisplatin resistance, have not been shown to induce a similar resistance to oxaliplatin. A decreased likelihood of resistance development makes oxaliplatin a good candidate for first-line therapy. Studies also demonstrate additive and/or synergistic activity with a number of other compounds, however, suggesting the possible use of oxaliplatin in combination therapies [3].

Hepatocellular carcinoma (HCC) is characterized by increased proliferation and loss of physiological tissue homeostasis induced through mutations of tumor suppressor genes and defects in apoptosis signaling [4]. The loss-of-function mutation of the tumor suppressor gene p53 contributes to the decreased expression of CD95 and reduced sensitivity of HCC cells towards this apoptosis pathway [5]. Additionally, other apoptosis receptors (TRAIL-1 and -2) and cell cycle regulatory BH3-only domain proteins are decreased and antiapoptotic proteins are increased in HCC cells [6, 7]. HCC is one of the most common malignancies and the leading causes of cancer-related mortality, and it generally represents highly resistant features to chemotherapy [8-10]. Surgical resection and liver transplantation might be the most effective way to cure the cancer, but these approaches have limited applicability and advanced tumors frequently recur even after complete surgical resection [11, 12]. Hence, chemotherapy remains an important option to treat HCC and developments of new chemotherapeutic strategies are necessary.

Apoptosis is a highly preserved and controlled mechanism to achieve tissue homeostasis through targeted elimination of single cells without disrupting the biological functionality of the tissue. The morphological changes associated with apoptosis include nuclear condensation, cell shrinkage, and plasma membrane blebbing resulting in apoptotic bodies [13]. Rapid

engulfment of these through neighboring cells or macrophages prevents an inflammatory response as observed with necrotic cell death in most cases [14]. Apoptosis can be initiated through an extrinsic or an intrinsic pathway depending on the initial site of activation of the cell death process. The extrinsic pathway is initiated after binding of trimeric ligand molecules to corresponding preassembled receptor multimers [15, 16]. Most prominent cytokines to induce HCC apoptosis are the members of the tumor necrosis factor (TNF) ligand superfamily CD95L, TNF and TNF-related apoptosis-inducing ligand (TRAIL) [15]. These cytokines exert a physiological function through their cognate receptors, namely the CD95 receptor (Apo1/Fas receptor), TNF receptor type 1 (TNF-R1, p55/65, CD120a) and type 2 (TNF-R2, p75/80, CD120b), TRAIL receptor type 1 and type 2 [17]. Receptor interactions occur at the plasma membrane and result in conformational changes of the receptor, initiating the assembly of an early intracellular signaling complex, to which downstream signaling molecules are subsequently recruited [18]. The intracellular domains of death receptors are devoid of intrinsic kinase activity and therefore depend on hemophilic protein–protein interactions for the initiation of cell signaling [19]. The apoptotic cell death pathway is activated following recruitment of the Fas-associated death domain (FADD), which results in activation of the caspase (cysteine aspartate protease) family of enzymes [20]. The family can be divided into upstream initiator caspases, such as caspase-8, -9, -10 and downstream effector caspases, caspase-3 and -6. The effector caspases are responsible for the cleavage of proteins whose functional loss induces apoptosis [21]. Upon recruitment and co-localization with FADD, high local concentrations of procaspases-8 undergo autoproteolytic cleavage, releasing activated caspase-8. This complex has been termed the DISC, and the mode of activation is referred to as the induced proximity model of activation [22]. Expression of the FLICE inhibitory proteins (FLIP-long and -short) can block apoptosis at this point and prevent caspase-8 activation by

inhibition of the recruitment and processing of procaspases-8 at the level of the DISC [23]. The intrinsic pathway of apoptosis involves mitochondria and caspase-9 activation. Cleavage of the Bcl-2 family member Bid by caspase-8 results in truncated Bid (tBid), which triggers oligomerization of the proapoptotic Bcl-2 family members Bax and Bak [24]. These molecules then insert into the mitochondrial membrane, resulting in mitochondrial permeability transition (MPT) and release of mitochondrial proteins including cytochrome *c* [25]. The cytochrome c releases into the cytosol, triggers formation of the apoptosome, a complex with apoptosis protease-activating factor-1 and procaspases-9. Caspase-9 becomes activated and in turn activates caspase-3, leading to degradation of structural proteins and resulting in apoptosis [26].

The p53 protein has been denoted `the guardian of the genome' for its role in preventing the accumulation of genetic alterations. This task is achieved through the induction of growth arrest or senescence to prevent the replication of damaged DNA. However, the suppression of tumor development is largely dependent on the ability of p53 to eliminate aberrant cells by apoptosis [27-29]. The tumor suppressor p53 inhibits cell growth through activation of cell-cycle arrest and apoptosis [30], and most cancers have either mutation within the p53 gene or defects in the ability to induce p53. p53 is a critical cellular protein that plays an essential role in regulating the cell cycle and the cellular response to certain environmental or genotoxic stresses. Under normal growth conditions, p53 is a short-lived protein and is expressed at relatively low basal levels within the cell. In response to various cellular stresses, including exposure to DNA-damaging agents, UV and γ-irradiation, hypoxia, and nucleotide depletion, p53 is rapidly induced and functions as a transcriptional-dependent and –independent related apoptosis [31]. Several reports have suggested that translational regulation may also contribute to p53 induction after DNA damage. In the initial reports of p53 induction after ionizing irradiation, the protein-synthesis inhibitor cycloheximide was shown to block p53 induction,

and marked increases in labeling of p53 protein after treatment [32, 33]. Activation or re-introduction of p53 induces apoptosis in many tumor cells and may provide effective cancer therapy [34]. The apoptotic mechanisms of p53 have been intensively dissected and multiple pathways have been identified. This effort revealed the involvement of multiple apoptotic pathways. While p53-mediated growth arrest is dependent on its sequence-specific transactivation (SST) function, p53-promoted apoptosis is mediated by both SST-dependent and -independent pathways which cooperate for full apoptotic response [29, 35, 36].

p53 can activate the extrinsic apoptotic pathway through the induction of genes encoding transmembrane proteins Fas and DR5. The cell-surface receptor Fas, a member of the TNF-R family of receptors, is a key component of the extrinsic death pathway [37]. Fas appears to be dispensable for p53-dependent apoptosis [38, 39]. In addition to stimulating Fas transcription, overexpressed p53 may enhance levels of Fas at the cell surface by promoting trafficking of the Fas receptor from the Golgi. Thus, p53 can mediate apoptosis through Fas transport from cytoplasmic stores [40]. The second member of this receptor family is DR5/KILLER, DR5 is regulated by p53 in response to DNA damage [41] and in turn promotes cell death through caspase-8 [42]. The p53 family proteins (p53, p63, and p73) regulate apoptotic pathways upstream of caspases in response to genotoxic drugs through transcriptional activation of proapoptotic genes, the products of which participate in the major apoptotic pathways: TNF receptor superfamily members Fas and DR5 in the death receptor pathway, and pro-apoptotic Bcl-2 family members (Bax, Puma, Noxa and Bid) in the mitochondrial pathway [43, 44].

One of the key proteins that modulates the apoptotic response is NF-kB, a transcription factor that can protect or contribute to apoptosis [45]. The mammalian NF-kB family contains 5 members, NF-kB1 (p105 and p50), NF-kB2 (p100 and p52), c-Rel, RelB, and RelA (p65). These proteins share a Rel homology domain (RHD), which mediates DNA binding,

dimerization, and interactions with specific inhibitory factors, the IKB, which retain NF-kB dimers in the cytoplasm. Many stimuli activate NF-kB, mostly through IKB kinase–dependent (IKK-dependent) phosphorylation and subsequent degradation of IKB proteins. The liberated NF-kB dimers enter the nucleus, where they regulate transcription of diverse genes encoding cytokines, growth factors, cell adhesion molecules, and pro- and antiapoptotic proteins [46, 47].

In other situations, however, NF-kB contributes to apoptosis [45]. NF-kB is activated by a variety of signals through mechanisms that result in phosphorylation and degradation of the inhibitory IKB proteins, the best understood being activation of IKB kinase IKK in response to TNFα signaling [48]. The NF-kB signaling pathway is regulated by the cytosolic-nuclear shuttling and modulation of the transcriptional activity [46]. The adapter protein TRAF2 and the kinase RIP1 play essential roles in NF-kB activation via TNF-R1 [49-52]. RIP is required for activation of the IKK complex [53]. The IKK complex phosphorylates IKB that forms and sequesters the NF-kB dimers in the cytosol. Immediately after phosphorylation, IKB undergoes ubiquitination and subsequent degradation by the 26S proteasome, which allows the translocation of the NF-kB dimers to the nucleus, where it activates a variety of target genes. Yeast two-hybrid screening and coimmunoprecipitation studies in mammalian cells have revealed that FLIP (L) interacts with TRAF1, TRAF2, and RIP [54, 55]. p53 stabilization decreases upon NF-kB activation [56], under special circumstances apoptosis induced by p53 may involve activation of NF-kB. Induction of p53 causes due to an activation of NF-kB that correlates with the ability of p53 to induce apoptosis. Inhibition or loss of NF-kB activity abrogated p53-induced apoptosis, indicating that NF-kB is essential in p53-mediated cell death [57].

It has been recognized that one of the most important traits of biological membranes is their ability to form specialized localized domains with distinct composition and physical

properties [58]. Lipid rafts are low-density, detergent-resistant microdomains of plasma membrane that are enriched in cholesterol and glycosphingolipids. Caveolae, a subclass of rafts, are characterized by flask-like invaginations of the plasma membrane that are distinguished from bulk lipid rafts by the presence of caveolin-1. Rafts/caveolae are known to be abundant in various signaling molecules, such as cell surface receptors and intracellular signaling molecules, and thus, these microdomains have involved in many cellular functions, including the regulation of apoptosis and cell proliferation [59]. Growth factor receptors, T-cell receptors, and the tumor necrosis factor receptor superfamily have shown to interact with rafts/caveolae, and some intracellular signaling molecules are redistributed to rafts/caveolae after the activation of those receptors [60-64]. The disruption of rafts/caveolae results in the impairment of signaling events and receptor function. Therefore, it has been proposed that rafts/caveolae serve as molecular platforms that spatially organize appropriate molecules for specific signaling pathways [65].

Cholesterol is an abundant component of the plasma membranes of eukaryotic cells and plays an essential role in maintaining membrane integrity and fluidity [66]. It is also critical for liquid-ordered raft/caveolae formation by serving as a space between the hydrocarbons chains of sphingolipids [67, 68]. The depletion of cholesterol from the plasma membrane causes disruption of rafts/caveolae and release of raft/caveolae constituents into a non-raft/caveola membrane, which renders them nonfunctional [67, 69]. Functional activity of death receptors, such as Fas and DR5, requires their physical association with lipid rafts, which serve as plasma membrane platforms for death receptor–initiated signals in formation of efficient DISCs [70, 71].

In this study, we tried to examine the molecular mechanism of p53 expression and activation by oxaliplatin. Here we found that oxaliplatin-induced p53 by a transcriptional

mechanism including multiple death receptors dependent NIK/IKK/IKB/NF-kB activation pathway in HCC cells. In addition, we showed that, p53 translocated to plasma membrane by oxaliplatin and enhanced membrane raft-engaged death receptor activation. These results propose a positive feedback regulation between activation of death receptor and p53 in oxaliplatin-induced apoptosis, thus indicating that p53 contributes to regulation of an extrinsic death pathway.

CHAPTER 2: MATERIALS AND METHODS

2.1. Cell culture and drug treatment

HCC HepG2 and SK-Hep1 cells were maintained in DMEM medium and human colon cancer cell line HCT116 and lungs cancer A549 cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with heat-inactivated 10% fetal bovine serum (FBS; Invitrogen), 1% of 50 μg/ml penicillin and 50 μg/ml streptomycin (Welgene, Seoul, Korea) in a 37^{o}C humidified incubator in an atmosphere of 5%.

The cells were seeded at a density of 1.5 x 10^6 cells/10 cm^2 dish/7 ml medium, 5 x 10^5 cells/6 cm^2 dish/3 ml medium, 2 x 10^5 cells/3.5 cm^2 dish/1 ml medium 1 x 10^5 cells/well of 12-well plate/1 ml mediun and 1 x 10^4 cells/well of 96-well plate/100 μl medium then used for the drug exposure experiments after being culture overnight. Drugs treatment was performed by adding 50-100 μM oxaliplatin (L-OHP; Boryung Pharmaceutical, Seoul, Korea), to the culture medium and incubate for 24 h. Cells were pretreated for 1 h with 10 μM of a caspase inhibitors (z-IETD-FMK, z-VDA-FMK) or 0.5-2 μM MG132, 1-2 μM CHX, 10 ng TRAIL, or 1-3 mM MBCD (Sigma-Aldrich)

2.2. Morphological detection of apoptosis and necrosis by Hoechst 33342 and propidium iodide staining

2 x 10^5 cells were seeded in 3.5 cm^2 dishes per 1 ml of medium. After the overnight incubation, the indicated drugs were treated at various time points. Cells were incubated either with 1 μg/ml Hoechst 33342 and 5 μg/ml propidium iodide (PI) at 37^{o}C with 5% CO_2 for 15

minutes then both floating and attached cells were collected. The pooled cell pellets were washed with ice-cold phosphate-buffered saline (PBS), fixed in 4% formaldehyde on ice, washed again with PBS, resuspended and a fraction of the suspension was centrifuged in a cytospinner (Thermo Shandon, Pittsburgh, PA). The slides were air dried, mounted in an anti-fade solution and examined using a DM5000 fluorescence microscope (Leica, Wetzlar, Germany) at respective excitation/emission wavelengths of 340/425 nm (HO) and 580/630 nm PI. Morphological assessments of apoptosis and necrosis were performed, intact blue nuclei, condensed/fragmented blue nuclei, condensed/fragmented pink nuclei and intact pink nuclei were considered viable, early apoptotic, late apoptotic (secondary necrotic) and necrotic cells, respectively. Total of 500 cells from several randomly chosen fields were counted and the number of apoptotic and necrotic cells were expressed as a percentage of the total number of cells scored.

2.3. SDS-PAGE and Western blot analysis

For the detection of various kinds of protein levels, 5 x 10^5 cells were plated into the 6 cm^2 dish in 3 ml medium and incubated at 37^oC in 5% CO_2 contained humidified incubator overnight. At various time after incubated with drugs treatment, cells were collected and washed with 1X ice cold PBS. The collected cell pellets were lysed in lysis buffer (50 mM Hepes, 150 mM NaCl, 5 mM EDTA, 1% (v/v) Triton x-100, 20 mM NaF, 50 mM PMSF, 1 mM Na_3Vo_4, 100 µg/ml leupeptin and 10 µg/ml aprotinin). Cell debris was removed by centrifugation (13000 rpm, 15 minutes at 4^oC temperature). Protein concentration was measured by Bio-Rad protein assay and samples were boiled for 10 minutes with sample buffer. The boiled samples were loaded into a 8%, 10%, 12% or 15% SDS-PAGE gel according to the

molecular weight of proteins and electrophoresis was fixed at 50 Volts up to 30 minutes and increased by 100 V for 2 and half hours. Protein was electrophoretically transferred into 0.22 μm nitrocellulose transfer membrane. The transferred membrane was blocked for 1 h in 5% nonfat dry skim milk in 1X-TBST and incubated at 4^{o}C overnight with the primary antibodies to active caspase-3, IKK, p-IKK, Na^{+}/K^{+}-ATPase (Cell Signaling Technology, Danvers, MA), p53, p65, FADD, α-tubulin, Caveolin1, Fas, NIK, ERK2, IKB, Histone (Santa Cruz Biotechnology, Aanta Cruz, CA), caspase-8, RIP1, DR4, DR5 (ProScience). After washing 3 times in 1X-TBS with 0.1% Tween-20, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody and visualized with the super signal West pico chemiluminescence kit (Pierce Biotechnology, Rockfoed, IL) and signals were acquired by image analyzer (Image Station 4000MM, Kodak, NY).

2.4. RNA interference

To determine the role of p53, DR4, DR5, FAS, RIP1, FADD, p65, caspase-8, caspase-10, MAP4K4, MAP3K14, MDM2, FasL, TRAIL and scramble RNAs study. Various cells at 70% confluence in 60 mm dishes were transfected with 5-8 μg siRNA using the jetPEI reagent (Polyplus-Transfection, France) according to the manufacturer's protocol. The transfected cells were incubated for 36 h then exposed to drugs and whole experimental processes were completed in 72 h of post-transfection. The primers of siRNA are mentioned in Table 1.

Table 1. Primers for RNA interference

Gene	Primer	Sequence
p53	Sense	5’-CAC UAC AAC UAC AUG UGU A(dtdt) -3’
	Antisense	5’-UAC ACA UGU AGU UGU AGU G(dtdt)-3’
Fas	Sense	5’-GCU UAU ACA UAG CAA UGG U(dtdt)-3’
	Antisense	5’-ACC AUU GCU AUG UAU AAG C(dtdt)-3’
RIP1	Sense	5’-CAC ACA GUC UCA GAU UGA U(dtdt)-3’
	Antisense	5’- AUC AAU CUG AGA CUG UGU G(dtdt)-3’
FADD	Sense	5’-CCA AGA UCG ACA GCA UCG A(dtdt)-3’
	Antisense	5’-UCG AUG CUG UCG AUC UUG G(dtdt)-3’
DR4	Sense	5’-CUG GAA AGU UCA UCU ACU U(dtdt)-3’
	Antisense	5’-AAG UAG AUG AAC UUU CCA G(dtdt)-3’
DR5	Sense	5’-CAG ACU UGG UGC CCU UUG(dtdt)-3’
	Antisense	5’-UCA AAG GGC ACC AAG UCU G(dtdt)-3’
NF-kB	Sense	5’-CCU GAG CAC CAU CAA CUA U(dtdt)-3’
	Antisense	5’-AUA GUU GAU GGU GCU CAG G(dtdt)-3’
Control	Sense	5’-CCU ACG CCA CCA AUU UCG U(dtdt)-3’
	Antisense	5’-ACG AAA UUG GUG GCG UAG G (dtdt)-3’
Caspase-8	Sense	5’-GCU GCU CUU CCG AAU UAA U(dtdt)-3’
	Antisense	5’-AUU AAU UCG GAA GAG CAG C(dtdt)-3’
Caspase-10	Sense	5’-CUG UCU ACU CUU CGG AUG A(dtdt)-3’
	Antisense	5’-UCA UCC GAA GAG UAG ACA G(dtdt)-3’

MAP3K14	Sense	5’-CAG AAC UCC ACA AAC UGA U(dtdt)-3’
	Antisense	5’-AUC AGU UUG UGG AGU UCU G(dtdt)-3’
MAP4K4	Sense	5’-GAU AAG UUA CGU GUC UAC U(dtdt)-3’
	Antisense	5’-AGU AGA CAC GUA ACU UAU C(dtdt)-3’
MDM2	Sense	5’-GCU UGG CCU ACA GUC AUC U(dtdt)-3’
	Antisense	5’-AGA UGA AUG UAG GCC AAG C(dtdt)-3’
FasL	Sense	5’-CUC AGA CGU UUU UCG GCU U(dtdt)-3’
	Antisense	5’-AAG CCG AAA AAC GUC UGA G(dtdt)-3’
TRAIL	Sense	5’-CUC CUU GUA AAG ACU AUA G(dtdt)-3’
	Antisense	5’-CUA CAG UCU UUA CAA GGA G(dtdt)-3’

2.5. Caspase-8 and -3 activity assay

For the detection of caspase-8 and -3 activities, 2 x 10^5 cells were seeded in 3.5 cm^2 dishes per ml medium. After the time point of drug treatment, cells were collected by centrifugation. The collected cell pellets were lysed by 50 μl of chilled cell lysis buffer and incubated on ice for 10 minutes. Centrifugation of samples at 10,000 g for 1 minute at 4^oC in microcentrifuge, transferred the supernatant to a next tube and kept in ice. Protein concentration was measured by Bio-Rad protein assay methods. Protein samples were diluted at 300 μg to 50 μl by cell lysis buffer for each assay. 50 μl of 2x-reaction buffers (containing 10 mM DTT) and 5 μl of the 4 mM IETD-pNA substrate for caspase-8 and 4 mM DEVD-pNA for caspase-3 (200 μM final concentrations) were added to each sample and then incubated at 37^oC for one and half hours. 100 μl of each samples were transferred into the 96 well plates and read at 405 nm wavelength.

2.6. *In vitro* protein-lipid binding assay

Similar to the immunoblot analysis, phosphoinositide (PIP) and membrane lipid strips were pre-spotted with various indicated lipid species (p-M600, s-6000), commercially available from Echelon Bioscience Inc, Salt Lake City, UT. These strips contain 100 pmoles of various phospholipids spotted and immobilized on a nitrocellulose membrane. HCC cells were thoroughly rinsed with 1X-PBS after the drug incubated times before lysing with lysis buffer (50 mM Hepes, 150 mM NaCl, 5 mM EDTA, 1% (v/v) Triton x-100, 20 mM NaF, 50 mM PMSF, 1 mM Na_3Vo_4, 100 μg/ml Leupeptin, and 10 μg/ml protinin) and freshly prepared 1X protease inhibitor cocktail. The nitrocellulose strips were blocked in 1X-TBST 3% fat-free BSA (Sigma, Saint Louis, MI) for 1 hour at room temperature. Then 100 μg/ml proteins from cell lysate was incubated with the strips in TBST 3% fatty acid free BSA at 4^{o} C overnight. The strips were then washed with 1X-TBST three times with gentle agitation for 10 minutes each wash at room temperature. Protein interaction with spotted phospholipids were detected by subsequently incubating the strips in a 1:1000 dilution of primary antibodies p53 (Santa Cruz Biotechnology) in a blocking buffer for 2 h and then membranes were washed with 1X-TBST three times for 10 minutes each at room temperature. Membranes were incubated with HRP-conjugated secondary antibody for an additional hour after thoroughly washed with 1X-TBST and visualized with Super Signal Chemiluminescence kit (Pierce Biotechnology) and finally the signals were acquired by image analyzer (Image station 4000MM, Kodak, NY, USA).

2.7. Luciferase activity assay

HepG2 and SK-Hep1 cells were plated at 7 x 10^4 cells per well in 12-well plates 24 h prior to the transfection. Cells were transfected using the interferin transfection reagent (Bioparc)

with 1 µg pNF-kB metluc2 reporter vector (Clontech laboratories, Inc.) or combination with 0.1 µg siRNA p53 or siRNA DR4 or siRNA DR5 or siRNA Fas. The reporter vector was designed to monitor the NF-kB signal transduction pathway in cells by quickly sampling the media supernatant. The vector promoter activity can be directly correlated to the amount of metridia luciferase secreted in the medium. After the 24 h of transfection, cells were treated with 50 µM oxaliplatin, 1-3 mM MBCD for 30 mins, 1 h and 4 h. After the indicated times of drug incubation, 50 µl of supernatant was transferred into the 96 well plates (Nunclon^{TM}) then added 5 µl of 1x-luciferase substrate into each well and detected by luminometer.

2.8. Immunofluorescence

siRNA control and siRNA p53 transfected cells were cultured on coverslips in 24 well plates, one night before the experiments. Cells were treated with 100 µM oxaliplatin for 24 h. Cells were fixed with 1% formaldehyde about 15 minutes and rinsed with 1X-PBS and then permeabilized with 0.2% triton x-100/PBS for 10-15 minutes, blocking of cells with 3% BSA/PBS for 1 h at room temperature. Cells were incubated with anti p53 antibody 1:1000 dilution in BSA at 4^0C overnight. Cells were then stained with nuclear staining Hoechst 2 µg/ml and membrane raft staining with cholera toxin B 10 µg/ml (1:100 dilutions in PBS) for 15 minutes. Coverslips were fixed into the slides after washing 2 times with 1X-PBS and one time with water then fixed with mounted gel. Slides were observed under a confocal microscope.

2.9. Subcellular protein fractionation

For subcellular protein fractionation, HepG2 and HCT116 cells were plated in 10 cm^2 cultural dishes with 1.5 x 10^6 cells per 7 ml medium. After the overnight of incubation in 37^0C in 5% CO_2 incubator, drugs were treated for 24 h. Cytosolic protein (fraction 1-C), membrane/organelle protein (fraction 2-M), nuclear protein (fraction 3-N) and cytoskeleton protein (fraction 4-S) were extracted by using ProteoExtract subcellular proteome extraction ® kit (Calbiochem) according to the manufacturer's protocol. Anti α-tubulin, Na^+/K^+-ATPase and histone was used as a marker for cytosolic, membrane and nuclear proteins respectively.

2.10. Quantitative real-time PCR

For the measurement of transcription level, total cellular RNA was isolated from cells using TR1 reagent (RNAisoPlus, Takara) according to the manufacturer's instructions. cDNA for each RNA sample was synthesized in 20 µl reactions using the superscript first strand synthesis system for reverse transcription PCR (Invitrogen, USA) following manufacturer's protocol. PCR reaction contained 4 µl of 1:5 diluted cDNA, 4 mM $MgCl_2$ 10 pmole of each primer and 4 µl first Starter Mix buffer (dNTPs, SYBER Green dye and Tag polymerase). For verification of the correct amplification product, PCR products were analyzed on a 2% agarose gel, stained with ethidium bromide. Primer sequences were as follows: for **p53** 5'-ACT AAG CGA GCA CTG CCC AAC-3' (S), 5'-CCT CAT TCA GCT CTC GGA ACA TC-3' (AS); **β-actin** 5'-GAC TAT GAC TTA GTT GCG TT-3' (S), 5'-GTT GAA CTC TAC ATA CTT CCG-3' (AS). Melting curve analysis was performed to confirm the production of a single product. Negative control without template was produced for each run. Data were analyzed

using Light Cycler software version 4.0 (Roche Switzerland).

2.11. Lipid raft and non-raft fractionation and protein precipitation

Lipid rafts and non-rafts soluble fractions were separated by discontinuous sucrose density gradients from treated and untreated cells. Cells from 15 cm^2 cultural dishes (1 x 10^7 cells/dish/18 ml medium) were collected by scraping method, lysed on room temperature for 30 minutes in 1 ml of lysis buffer (1% Brij35 in 25 ml Hepes, 1 mM EDTA, and 150 mM NaCl [6.5 pH]) supplemented with protease inhibitor cocktail (Complete tablets; Roche), and then homogenized with a loose-fitting glass homogenizer. Homogenates were mixed with 1 ml of 80% sucrose made with Hepes buffer (25 mM Hepes, 1 mM EDTA, and 150 mM NaCl [6.5 pH]) and then placed on the bottom of a centrifuge tube (14 x 89 mm, Backman). The samples were then overlaid with 6.5 ml of 30% sucrose and 3 ml of 5% sucrose and centrifuged at 37,000 rpm using SW-41 rotor in a Beckman ultracentrifuge for 18 h at 4^oC. Twelve fractions of about 1 ml was collected from top to bottom of the gradients and made aliquot.

The protein in the sample was precipitated for concentration and sucrose removal. 800 µl of methanol was added into the 200 µl of a protein fraction in a 2 ml eppendorf, and tube then vertexes. 200 µl chloroform was mixed and then vortexes, 600 µl water was added and then vortexes again (appears cloudy white). Immediately centrifuge for 10 minutes at full speed in a microcentrifuge. A white disc of protein was discarded and added 867 µl of methanol to the tube and inverted about 5 times. Again spin for 10 minutes at full speed in a microcentrifuge. All liquid was removed and allowed the pellet to air dry. The precipitated protein pellet was dissolved in 4x-sample buffer and subjected to Western blot for detection of pro-apoptotic proteins [72].

2.12. Protein qualification

As following the Bio-Rad protein assay, the standard curve was set up with BSA dilution in water as final concentration of 0, 1.56, 3.125, 6.25, 12.5 and 25 µg/µl. 2.5 µl of each sample were added with 1 ml of Bio-Rad protein assay reagent (1:4 dilutions with 3 DW) vortexes and mixed well. 200 µl of each sample mixture was moved into 96 well plates. Light absorbance was read at 595 nm, compared to the standard curve to determine the protein concentration on the samples [73].

2.13. Statistical analysis

All data were analyzed and presented as mean ± SD from the number of independent experiments. Statistical analysis was performed by Student's *t*-test at a significance level of $p < 0.05$.

CHAPTER 3: RESULTS

3.1. p53 is necessary for oxaliplatin-induced apoptosis

p53 mediates the cellular response to various stresses by activating different downstream effectors, depending on the type of cell and the nature of the cellular stress. It is widely accepted that p53 exerts tumor suppression activity by regulating the transcription of several genes that are involved in the control of processes such as cell growth and apoptosis. p53 can induce apoptosis by stimulating the extrinsic death-receptor pathway or, more importantly, the intrinsic mitochondrial pathway [32]. Transcriptional-dependent and -independent mechanisms of p53 might lay the foundations of new therapeutic approaches. Here we tried to investigate the transcription-independent mechanism of p53.

Oxaliplatin induces apoptosis in HepG2 cells through p53 transcription-independent mechanism. Oxaliplatin triggered cell death in different cell types such as HCT116, A549, SNU601 as well as HepG2 cells. Thus, we analyzed the role of p53 in apoptosis by oxaliplatin treatment. Cells were transiently transfected with siRNA control and siRNA p53 for 36 h and then exposed to 25-50 μM oxaliplatin for 24 h. Collected cells were examined for cleaved caspase-3, p53 and α-tubulin by Western blot analysis. Oxaliplatin-induced cleaved caspase-3 was suppressed with interference of p53. As same conditions, cells were subjected to analyze for caspase-3 activity assay. The activation of caspase-3 was decreased in the interference of p53 in response to oxaliplatin (Fig. 2). These results indicate that p53 is required for oxaliplatin-induced apoptosis.

We investigated the effect of oxaliplatin on p53 expression. For these experiments, the well established wild-type p53-harboring HepG2, SK-Hep1, A549, HCT116 as well as p53 mutant SNU601 and SNU423 cells were treated with 100 μM oxaliplatin as the function of times. The level of p53 proteins were significantly induced as early 3 h after treatment of oxaliplatin in wild-type p53-harboring cells but p53 mutant cells has no significant effect (Fig. 3). Maximum induction of p53 protein was observed at 24 h after oxaliplatin exposure. In contrast, the levels of control housekeeping protein α-tubulin was unchanged. A real-time PCR analysis was done to determine whether the increase in p53 protein was associated with a corresponding change of p53 mRNA levels. The levels of p53 mRNA were increased as the function of times i.e. 6 h, 12 h and maximum 18 h after oxaliplatin treatment. The p53 mRNA levels were highly increased in HepG2 and SK-Hep1 cells but not significance in A549 and HCT116 cells (Fig. 3).

To determine the p53 expression pathway, four kinds of wild-type p53-harboring cells were treated with oxaliplatin in the presence or absence of protein synthesis inhibitor cyclohexamide for 18 h and 24 h. Treated and untreated cells were collected and analyzed by Western blot for p53 and α-tubulin. Oxaliplatin-induced p53 expression was suppressed by cyclohexamide combination mainly in HepG2 and SK-Hep1 cells (Fig. 3).

To confirm the p53 expression pathway, 4 kinds of cells were pre-treated with protein synthesis inhibitor cyclohexamide and proteosomal protein degradation inhibitor MG132 in the presence or absence of 100 μM oxaliplatin for various durations. Treated and untreated (control) cells were collected and examined by Western blot analysis for p53 levels and α-tubulin. Oxaliplatin-induced p53 expression was suppressed with cyclohexamide combination, similar pattern in all 4 kinds of cells. The proteosomal protein degradation inhibitor MG132 did

not suppress p53 expression levels induced by oxaliplatin. For more evidence, HepG2 cells were transiently transfected with siRNA control and siRNA MDM2. After 36 h of transfection, cells were pre-treated with cyclohexamide in the presence or absence of oxaliplatin for 24 h. From the Western blot analysis, oxaliplatin-induced p53 expression was suppressed by CHX but not by interference of MDM2 (Fig. 4). These results indicate that oxaliplatin induces p53 expression in wild-type p53-harboring cells through protein synthesis pathway but not by proteosomal protein degradation.

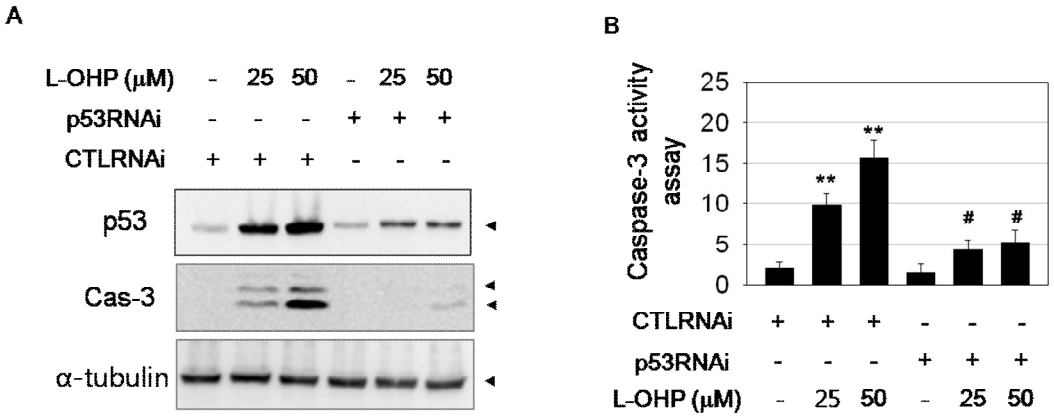
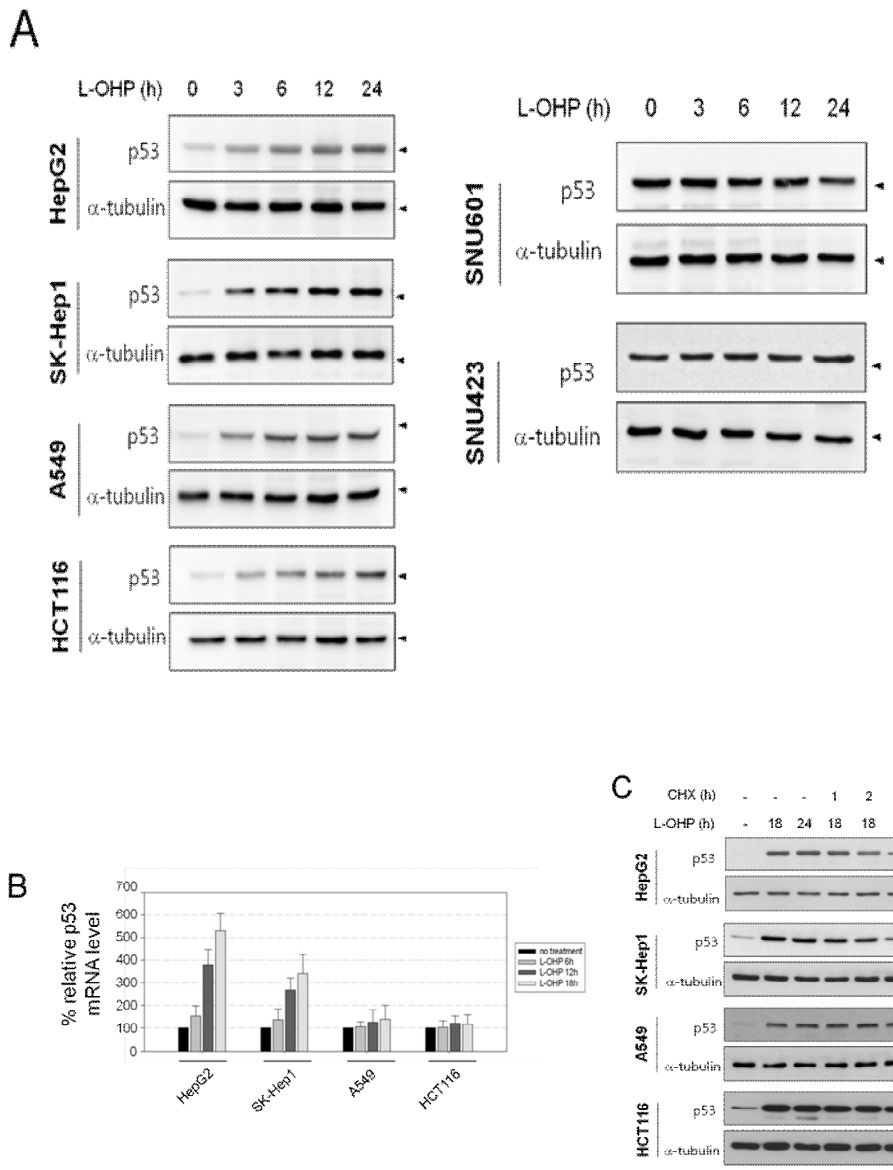


Figure 2. Role of p53 in oxaliplatin induces apoptosis. HepG2 cells were transiently transfected with scramble RNAi(CTLRNAi) or p53 RNAi for 36 h and then cells were exposed to 25-50 μM oxaliplatin (A and B). After 24 h incubation, cells were collected and analyzed by Western blot for p53, cleaved caspase-3 and α-tubulin (loading control) (A). or subjected for caspase-3 activation by measuring DEVD-pNA substrate caspase-3/CCP32 colorimetric assay kit (B). $^{**}p < 0.01$, $^{\#}p < 0.05$.


A
L-OHP (h) 0 3 6 12 24
HepG2
p53
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SK-Hep1
A549
HCT116
SNU601
SNU423
B
% relative p53 mRNA level
700
600
500
400
300
200
100
0
no treatment
L-OHP 6h
L-OHP 12h
L-OHP 18h
HepG2
SK-Hep1
A549
HCT116
C
CHX (h) - - - 1 2 6
L-OHP (h) - 18 24 18 18 18

Figure 3. Oxaliplatin induces p53 expression in wild-type p53-harboring cells. (A) HepG2, SK-Hep1, SNU 423, HCT116, A549 and SNU-601 cells were treated with 100 μM oxaliplatin for (3, 6, 12 and 24 h) time dependent manner, then cells were collected and examined by Western blot for p53 expression and α-tubulin. (B) HepG2, SK-Hep1, A549 and HCT116 cells were incubated in cultured medium with 100 μM oxaliplatin for 6, 12 and 24 h. After the drug incubation, total RNA was extracted from the cells then changed into cDNA by reverse transcriptase PCR. The cDNA product was used for examined the p53 relative mRNA by real-time PCR. (C) Cells were exposed with oxaliplatin for 18 and 24 h. 1 μM CHX was added 1, 2 and 6 h before the completion of 18 h with oxaliplatin. The cells were collected and analyzed by Western blot for p53 and α-tubulin level.

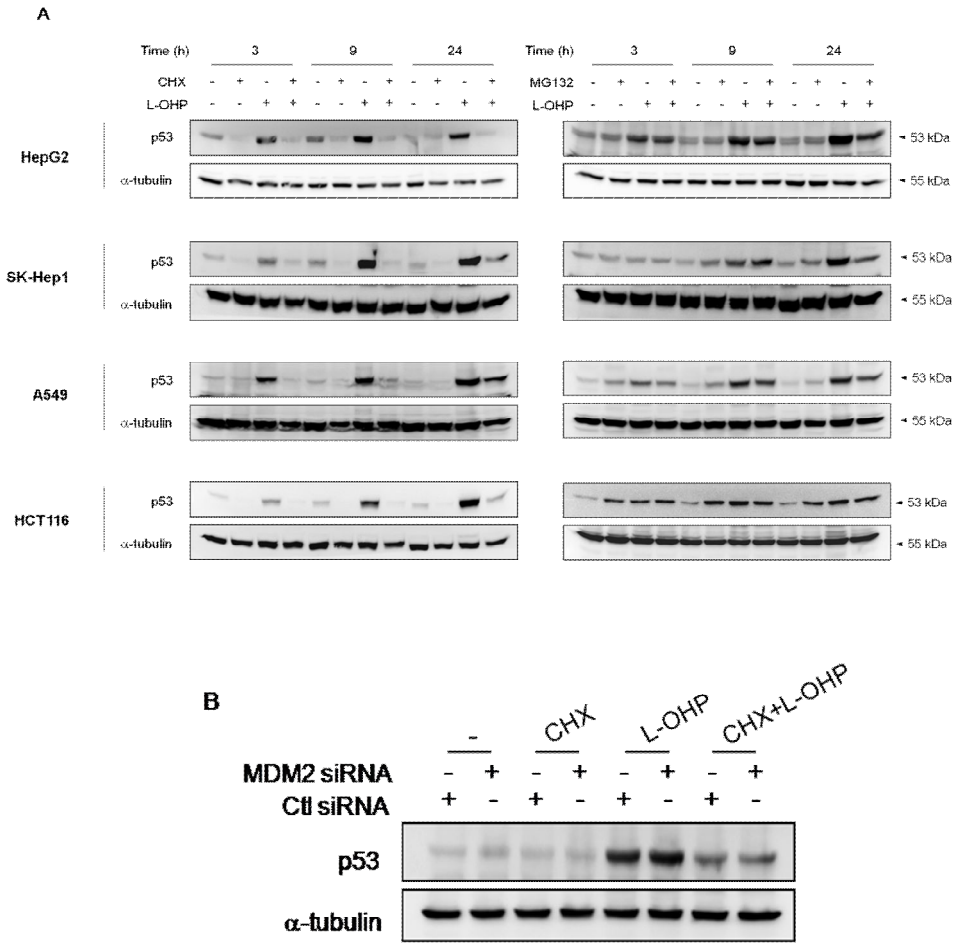


Figure 4. Oxaliplatin-induced p53 accumulation is not controlled by proteosomal protein degradation in HepG2 and SK-Hep1 cells. (A) Cells were pretreated by 1 μM CHX or 2 μM MG132 with or without 100 μM oxaliplatin for 3, 9 and 24 h. After the indicated time of incubation, cells were collected and analyzed for p53 and α-tubulin by Western blotting. (B) HepG2 cells were transiently transfected with siRNA control or siRNA MDM2, after 36 h of transfection cells were pretreated with 1 μM CHX in the presence or absence of 100 μM oxaliplatin for 24 h. Cells were collected and then p53 and tubulin level was measured by immunoblotting.

3.2 Oxaliplatin induces p53 transcription in a NF-kB-dependent manner

The NF-kB subunit can contribute towards tumor cell survival through inducing the expression of a variety of antiapoptotic genes. However, the NF-kB response can show great diversity and not always antiapoptotic. NF-kB is a transcription factor that transcriptionally express the variety of genes including p53 [57]. Here we found that oxaliplatin induced p53 transcription in a NF-kB-dependent manner. HCC cells were transiently transfected with siRNA control or siRNA p65. After 36 h of transfection, cells were exposed with 100 μM oxaliplatin for 24 h. The treated and untreated cells were examined for p53 expression level by Western blot analysis. Oxaliplatin-induced p53 expression was suppressed with p65 interference in both types of cells. The transfection efficiency of p65 and loading control α-tubulin were measured. As the same time, p53 mRNA level was measured by real-time PCR, oxaliplatin-induced p53 mRNA level was decreased with p65 knockdown condition after 18 h of treatment (Fig. 5).

To further confirm the p53 transcription, both HepG2 and SK-Hep1 cells were treated with oxaliplatin as the function of times (0-9 h). Treated and untreated cells were analyzed by Western blot for p-IKKα, IKKα, IKBα, and α-tubulin. Oxaliplatin increased the p-IKKα and decreased the IKBα at early time 1 h to maximum 9 h. To investigate the NF-kB activation, cells were treated with oxaliplatin in various durations (0-4 h), then the culture medium (supernatant) was used to examine for NF-kB activation by metridia luciferase activity assay. Oxaliplatin induces NF-kB activation as early time point upto 4 h (Fig. 5). We would like to know if the p53 was transcriptionally expressed through NF-kB pathway. Cells were transfected with siRNA control and siRNA NIK i.e. MAP4K4 and MAP3K14. After 36 h of

transfection, cells were exposed with oxaliplatin for 24 h. Western blot result shows that the protein level of p53 and p-IKKα was suppressed by the interference of NIK in both types of cells. The p53 transcription level was measured in the NIK transfected cells after the indicated time of oxaliplatin treatment. Oxaliplatin-induced the level of p53 mRNA was significantly decreased by the interference of NIK (Fig. 5). These results indicate that oxaliplatin induces p53 transcription in a NF-kB-dependent manner.

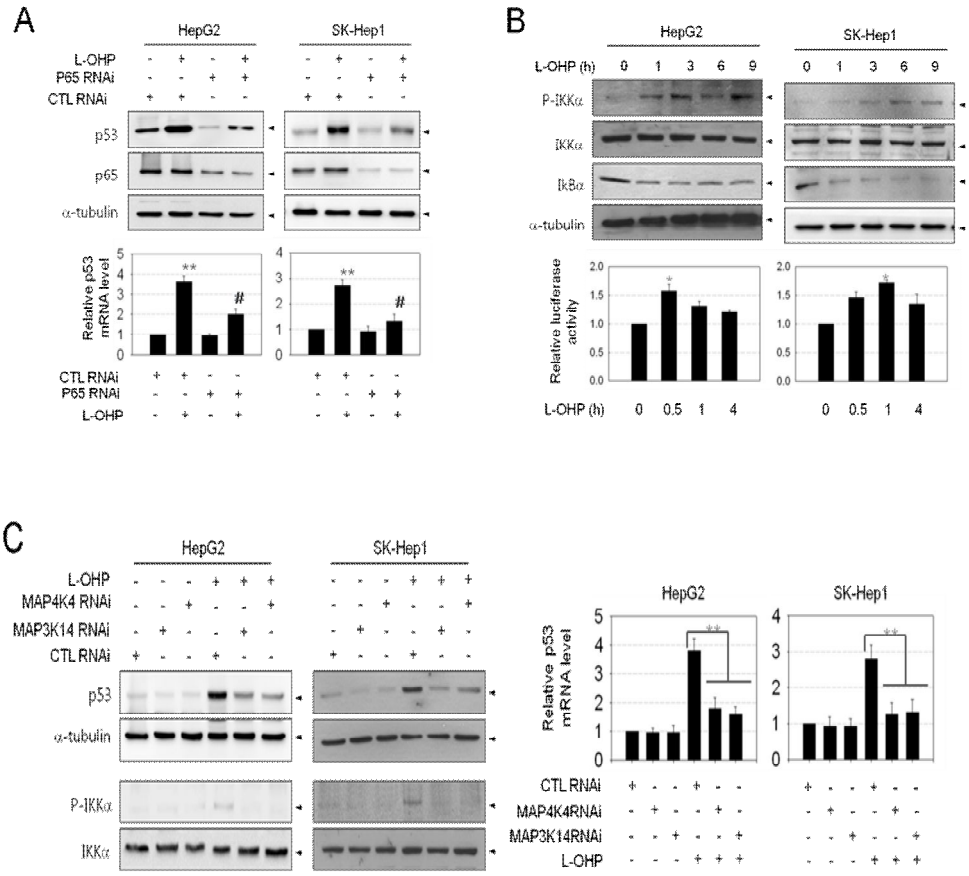


Figure 5. Role of NF-kB in oxaliplatin-induced p53 transcription. (A) HepG2 and SK-Hep1 cells were transiently transfected with siRNA control (CTL RNAi) or p65 RNAi for 36 h in cultured medium, then cells were exposed with 100 μM oxaliplatin. After 24 h of incubation, cells were collected and examined by Western blot for p53, p65 and α-tubulin. Relative p53 mRNA level was analyzed by real-time PCR. (B) Cells were treated with 100 μM oxaliplatin for various durations (1, 3, 6 and 9 h) and then phosphorylation of IKKα, IKKα, IKBα and α-tubulin levels were measured by immunoblotting. The NF-kB activation was anaylzed by luciferase activity assay. (C) Cells were transfected with siRNA NIK (MAP4K4 RNAi and MAP3K14 RNAi) and scramble RNAi (CTL RNAi) for 36 h, then cells were treated with oxaliplatin for 24 h. p53, p-IKKα, IKKα and α-tubulin levels were analyzed by Western blotting. mRNA of p53 was examined by real-time PCR. $^{**}p < 0.01$, $^{*}p < 0.05$, $^{\#}p < 0.05$.

3.3 Multiple death receptor signaling controls the NF-kB-dependent p53 expression

We show that p53 is expressed through the NF-kB-dependent pathway. Here we examined how the multiple death receptor signaling involved in NF-kB activation and p53 expression. For this, HCC cells were transiently transfected with siRNA control and various kinds of death receptor siRNA Fas, siRNA DR4 and siRNA DR5. After the 36 h of transfection, cells were treated with oxaliplatin as the function of times (0-4 h). Treated and untreated cells were analyzed by Western blot for the p53 expression level. Oxaliplatin-induced p53 expression level was significantly decreased by the interference of all 3 kinds of death receptors. The transfection level of Fas, DR4, DR5 and α-tubulin were measured in HepG2 and SK-Hep1 cells. Or cells were subjected to analyze for p53 mRNA level by real-time PCR. The p53 mRNA level was highly increased in siRNA control transfected cells after the oxaliplatin treatment and significantly suppressed by the interference of death receptor (Fig. 6). These results indicate that oxaliplatin induces p53 expression through the multiple death receptor and NF-kB signaling pathway.

To confirm this result, cells were transfected as mentioned before, then treated with oxaliplatin as the function of times (0-4 h) and analyzed the NF-kB activation again by metridia luciferase activity assay. The NF-kB was activated maximally in siRNA control transfected cell at 1 h and started to decrease after 4 h of oxaliplatin exposure. NF-kB activation was blocked by the interference of death receptors in HepG2 and SK-Hep1 cells (Fig. 7). These results confirm that the multiple death receptors are involved in NF-kB-dependent p53 expression in response to oxaliplatin.

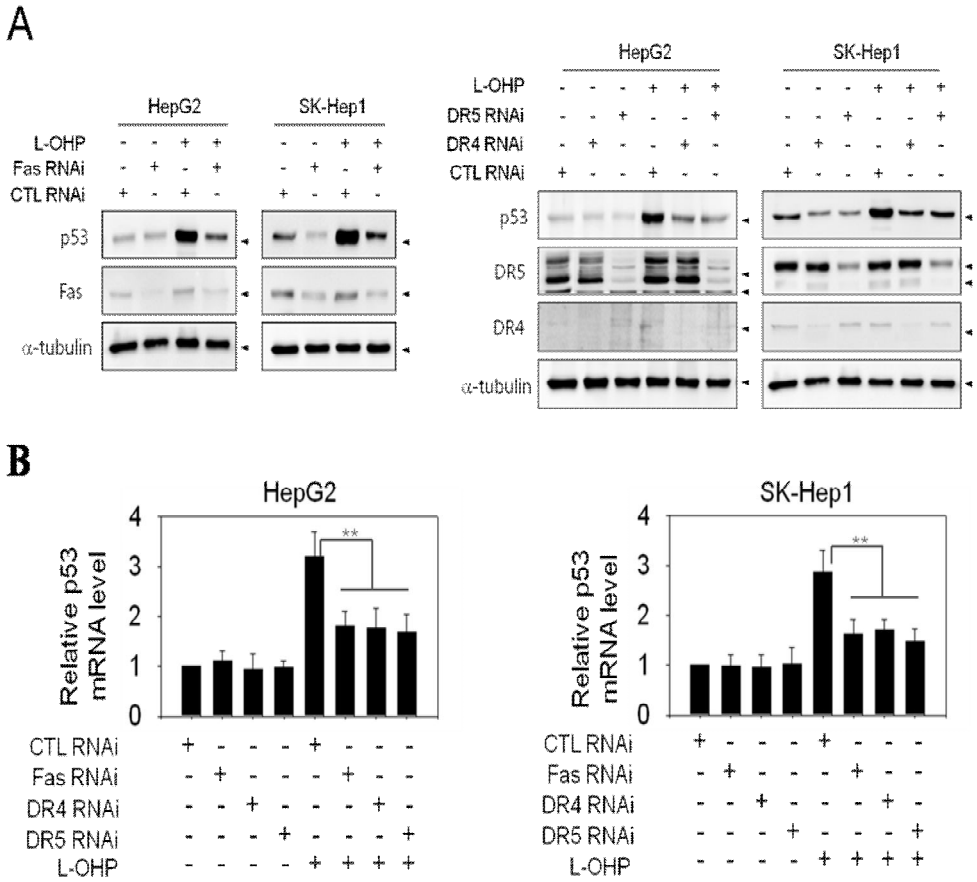


Figure 6. Involvement of multiple death receptors in p53 expression. (A) HepG2 and SK-Hep1 cells were transfected with various death receptor, Fas RNAi, DR4 RNAi, DR5 RNAi and CTL RNAi. After the 36 h of transfection, cells were treated with 100 μM oxaliplatin for 24 h. The p53 expression level and transfection efficiency of Fas, DR4 and DR5 were measured by Western blot analysis method, α-tubulin (loading control). (B) Cells were transfected and treated as mentioned in (A), and then relative p53 mRNA was analyzed by real-time PCR. ***p* < 0.01.

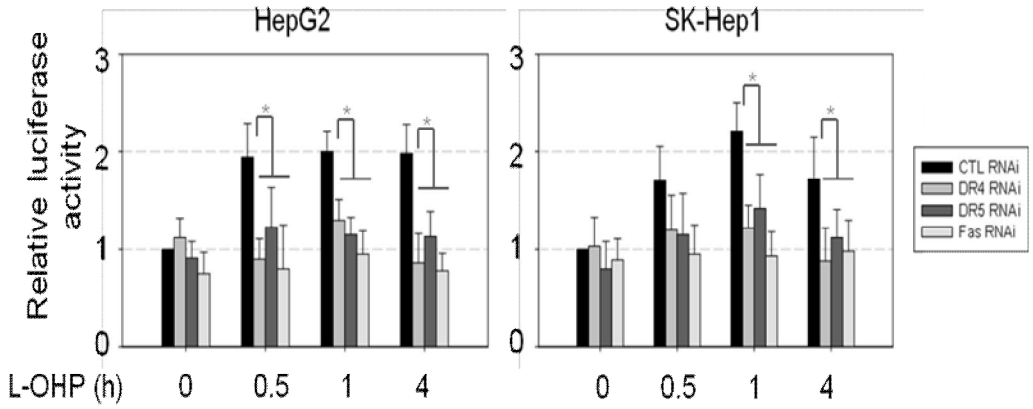
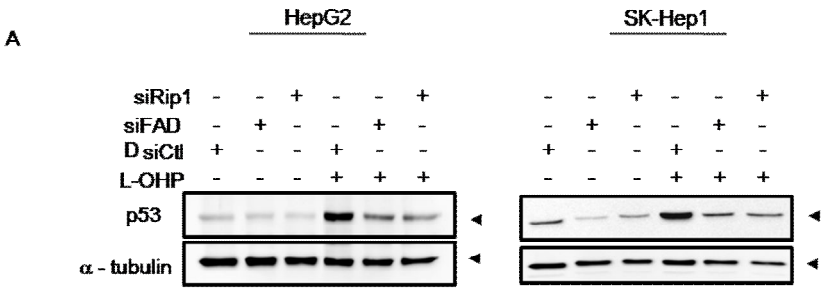


Figure 7. Involvement of multiple death receptors in NF-kB activation. HepG2 and SK-Hep1 cells were transfected with scramble RNAi (CTL RNAi), or DR4 RNAi, DR5 RNAi and Fas RNAi and then cultured for 36 h. Cells were exposed with 100 μM oxaliplatin for various durations (0.5, 1 and 4 h). *$p < 0.05$.

3.4. Oxaliplatin-induced p53 expression is regulated through multiple death receptor pathway

Caspase-8 and -10 have been mainly characterized as initiator caspases in the death receptor signaling to apoptosis. Recruitment of caspase-8 and -10 to the DISC, which is assembled upon triggering of several death receptors, such as TNF receptor 1, Fas and TRAIL, induces pro-caspase homodimerization that is followed by autoproteolytic cleavage [74]. Apart from their well-known function in apoptosis, caspase-8, -10 and c-FLIP have also implicated in NF-kB activation. Initial studies demonstrated that overexpression of these proteins in HEK293T cells induces NF-kB activation, which depends on their DED in the N-terminal prodomain [75, 76]. Caspase-8 and -10 have been shown to co-immunoprecipitate with RIP1, NIK, and IKKs upon overexpression in HEK293T cells [75, 77] providing a possible mechanistic link. *In vitro* pull-down assays with recombinant proteins showed a direct interaction with RIP1, NIK and TRAF2, whereas the binding of IKK is an indirect effect [78]. In the previous study, we found that death receptors regulated the p53 expression in response to oxaliplatin. Here we examined the involvement of death domains FADD and Rip1 in p53 expression. Adaptor proteins FADD and Rip1 were inhibited by their RNAi in HepG2 and SK-Hep1 cells and then treated with 100 μM oxaliplatin for 24 h. Treated and untreated cells were analyzed by Western blot for p53 expression level. In the siRNA control transfected cells, oxaliplatin-induced p53 expression was suppressed by the interference of FADD and Rip1. The interaction of caspase-8 and -10 with adaptor molecule was checked in response to oxaliplatin. For that, HCC cells were transfected by siRNA control, siRNA caspase-8 and -10, after 36 h of transfection cells were treated with oxaliplatin for 24 h. The p53 expression level was suppressed by the interference of caspase-8 and -10 after oxaliplatin exposure. The transfection efficiency of caspase-8 and

loading control were measured by Western blot analysis (Fig. 8). To determine the role of caspase-8 and pan-caspases, cells were pretreated with 10 μM caspase-8 inhibitor (Z-IETD) and pan-caspases inhibitor (Z-VAD) in the presence or absence of 100 μM oxaliplatin for 24 h. Cells were collected and analyzed by Western blot for p53 level. Caspase-8 and pan-caspases inhibitor did not suppress the p53 expression (Fig. 9). These results indicate that caspase-8 catalytic activity in signal transduction through FADD, Rip1, NIK, IKK and IKB contributes to NF-kB activation, but NF-kB activation after NIK and IKK signaling might be independent of caspase-8.



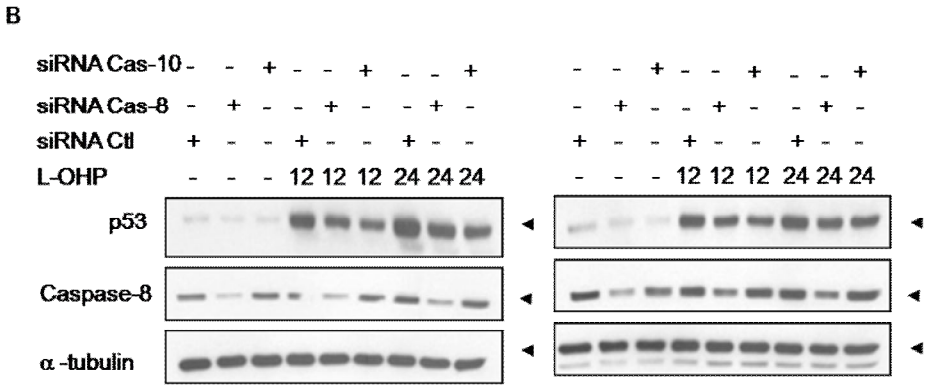


Figure 8. Oxaliplatin-induced p53 expression is regulated through multiple death receptor pathway. (A) HepG2 and SK-Hep1 cells were ransiently transfected with siRNA control, siRNA FADD, siRNA Rip1 and siRNA caspase-8, and -10. After 36 h of transfection, cells were treated with 100 μM oxaliplatin for 24 h. p53 expression and transfection efficiency of caspase-8 (B) was measured by Western blot analysis. α-tubulin (loading control).

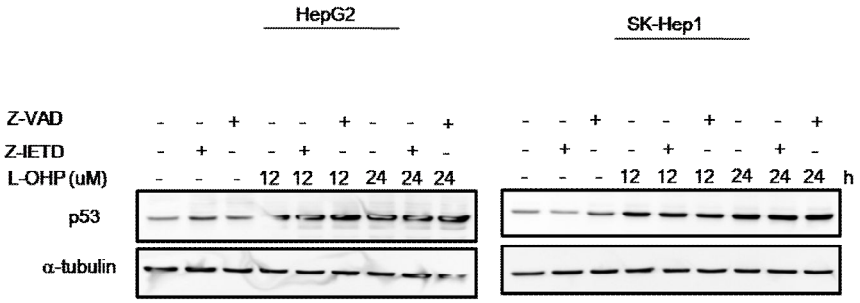


Figure 9. Caspase-8 and pan-caspases are not involved in oxaliplatin-induced p53 expression. HepG2 and SK-Hep1 cells were pretreated with 10 μM caspase-8 inhibitor (z-IETD) and pan-caspases inhibitor (z-VAD) in the presence or absence of 100 μM oxaliplatin for 24 h. Cells were then collected and analyzed for p53 protein level by immunoblotting.

3.5. FasL and TRAIL is not involved in oxaliplatin-induced p53 expression

TRAIL activates the extrinsic apoptosis pathway in most tumor cell types. TRAIL binding to its cognate agonistic receptors DR4 and DR5 induce the formation of the death-inducing signaling complex, a multimolecular platform that allows the activation of the initiator procaspases-8 and -10, which triggers the proteolytic caspase cascade leading to apoptosis [79]. FasL is a member of TNF ligand family and plays a major role in the induction of apoptosis through the binding to its agonistic receptor Fas [80]. In our study, oxaliplatin-induced p53 expression has regulated by death receptors, such as Fas, DR4 and DR5. FasL and TRAIL triggers the receptor activation, so we examined the agonistic effect of FasL and TRAIL on oxaliplatin-induced p53 expression. HepG2 and SK-Hep1 cells were transfected with siRNA FasL and TRAIL, then exposed with oxaliplatin. In the knockdown of FasL and TRAIL, p53 expression level was not changed. For confirmation, HepG2 cells was treated with 10 ng/ml TRAIL in the presence or absence of (25–50 μM) oxaliplatin for 24 h, then cells were collected and analyzed for the p53 expression level by Western blotting. TRAIL did not increase the oxaliplatin-induced p53 expression (Fig. 10). These results indicate that oxaliplatin-induced p53 expression is not regulated by FasL and TRAIL.

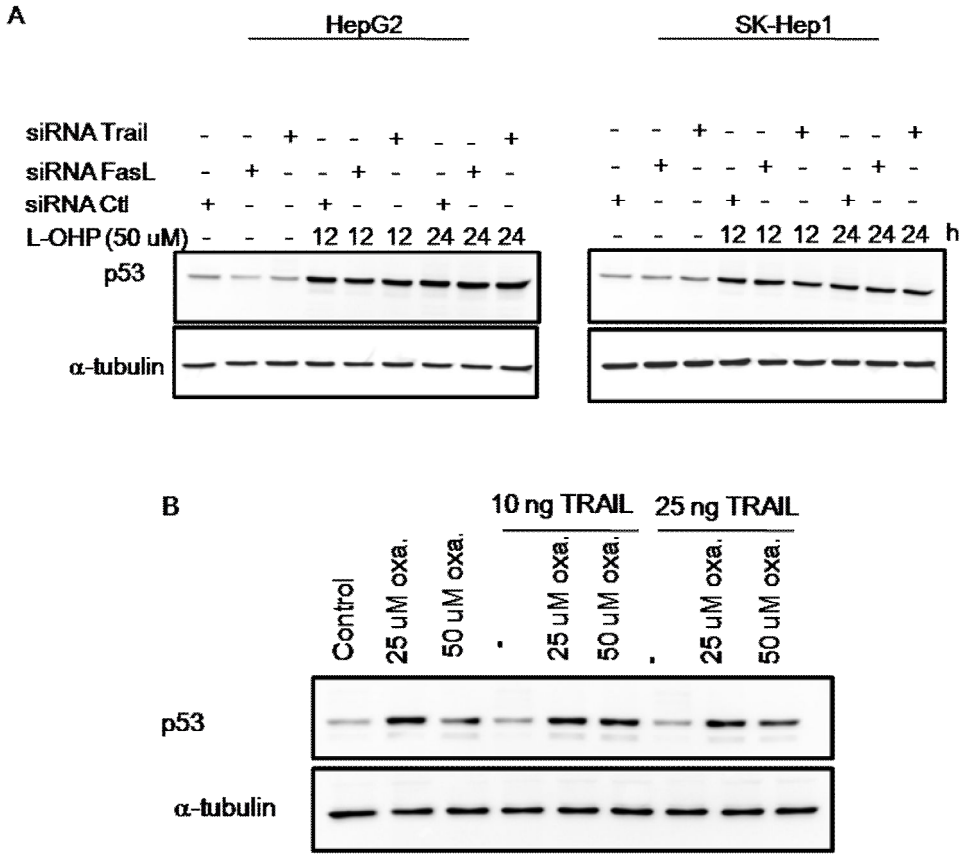


Figure 10. Oxaliplatin-induced p53 expression is not regulated by FasL and TRAIL. (A) 100 μM oxaliplatin was treated after the 36 h of transfection of siRNA control, siRNA FasL and TRAIL in HepG2 and SK-Hep1 cells. Cells were collected by centrifugation and p53 level analyzed by Western blot. (B) 10 ng/ml and 25 ng/ml TRAIL was treated with and without 25 μM and 50 μM oxaliplatin for 24 h in HepG2 cells. Treated and untreated cells were used for the measurement of p53 and α-tubulin level by Western blot analysis.

3.6. Oxaliplatin-induced apoptosis is dependent on multiple death receptors

Apoptosis is a normal cellular process involving physiologically relevant cell death. Apoptosis is initiated and promoted by a wide variety of intra- and extra-cellular stimuli. Extra-cellular induced apoptosis can be triggered by cell surface “death” receptors that possess cytoplasmic death domains FADD and TRADD, typified by members of the TNFR-1 [81]. We examined whether oxaliplatin-induced apoptosis was regulated by Fas, DR4, DR5 or death receptors ligand FasL and TRAIL in HepG2 cells. Cells were inhibited the expression of Fas, DR4, DR5, FasL and TRAIL by their siRNA, After 36 h, cells were treated with 100 μM of oxaliplatin for 24 h. Apoptotic cell death were examined by Hoechst staining from the fluorescence microscope. Oxaliplatin-induced apoptosis was significantly suppressed by the interference of death receptors Fas, DR4 and DR5 but ligands of these receptors FasL and TRAIL did not decrease the apoptotic bodies. Or cells were subjected for caspase-8 activation by measuring IETD substrate cleavage by FLICE/caspase-8 colorimetric assay kit. Caspase-8 activation was significantly decreased in the Fas, DR4 and DR5 knockdown cells, when blocked the FasL and TRAIL, caspase-8 activiation was not decreased (Fig. 11). These results indicate that, death receptors are involved in oxaliplatin-induced apoptosis in HCC cells. FasL and TRAIL did not trigger in oxaliplatin-induced apoptosis. Furthermore confirmation, cells were transfected by siRNA of death receptors Fas, DR4 and DR5 and then treated with oxaliplatin for 24 h. Treated and untreated cells were used in Western blot to analyze for cleaved caspase-3. Oxaliplatin-triggered cleavage of caspase-3 was decreased by the interference of Fas, DR4 and DR5. Next, to clarify whether oxaliplatin effect was specific to increase the level of death receptor, cells were treated with oxaliplatin for 24 h. The expression levels of Fas, DR4 and DR5 were examined by Western blot analysis. The death receptors level

did not change in response to oxaliplatin (Fig. 12). So death receptors are only involved in expression of p53 without changing their protein levels.

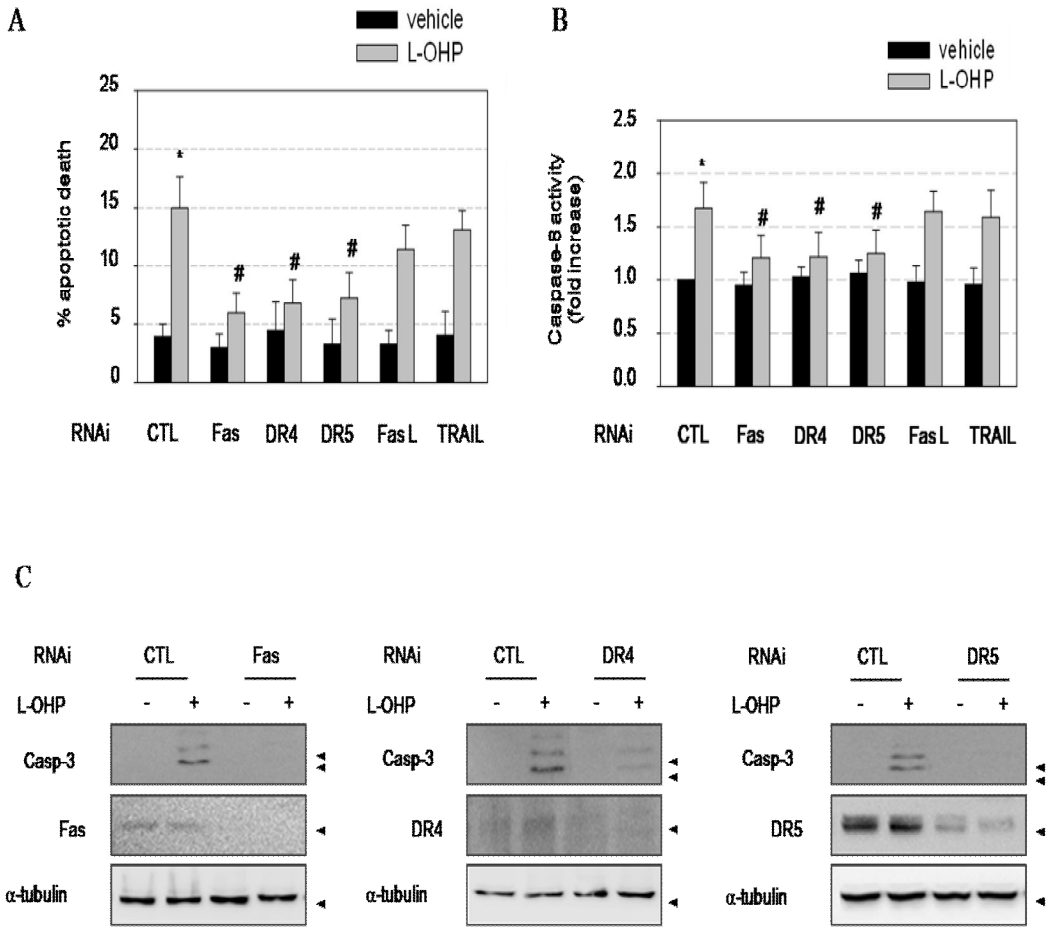


Figure 11. Involvement of multiple death receptors in oxaliplatin induces apoptosis. (A) HepG2 cells were transiently transfected with scramble RNAi, RNAi Fas, RNAi DR4, RNAi DR5 (A, B and C) and RNAi FasL and RNAi TRAIL (A and B). After 36 h of transfection, cells were treated with 100 μM oxaliplatin or untreated (control) for 24 h. Cells were then stained with HO/PI double staining to identify apoptotic cells. Cells with condensed or fragmented nuclei were counted as apoptotic cells under fluorescence microscope. (B) Caspase-8 activation was assessed by measuring IETD-pNA substrate cleavage by FLICE/caspase-8 colorimetric assay kit. (C) Transfected cells were treated with 100 μM oxaliplatin for 24 h and then examined for cleaved caspase-3 by Westerm blot, the transfection efficiency of Fas, DR4 and DR5 were detected. $^{*}p < 0.05$, $^{\#\#}p < 0.01$.

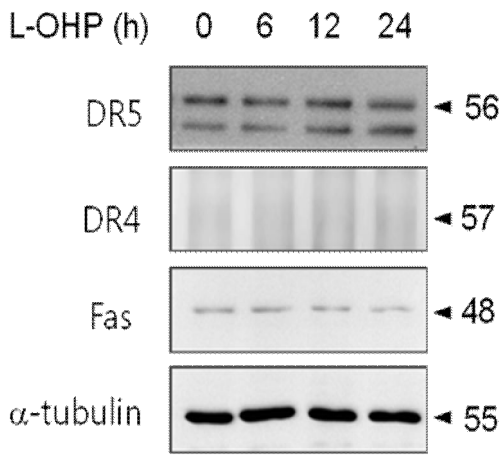


Figure 12. Oxaliplatin treatment does not increase protein levels of death receptors. HepG2 cells were treated with 100 μM oxaliplatin for various durations (6, 12 and 24 h). Treated and untreated cells were collected and analyzed by Western blot for DR5, DR4, Fas and α- tubulin.

3.7. Apoptosis-inducing proteins are localized at lipid rafts fraction in oxaliplatin-induced apoptosis

Cell membranes contain a variety of lipid species that differ in their physico-chemical properties. Lipid rafts are proposed to be highly dynamic, submicroscopic assemblies that float freely within the liquid disordered bilayer in cell membranes and can coalesce upon clustering of their components. Sphingolipids and cholesterol in the outer exoplasmic leaflet play a crucial role in the assembly of DISC proteins that triggers apoptosis [70]. Here we investigated the accumulation of p53 in membranous fraction in response to oxaliplatin. HepG2 and HCT116 cells were treated with 100 μM oxaliplatin for 24 h and then cytosolic, membranous, nuclear and cytoskeleton proteins were extracted by using ProteoExtract subcellular proteome extraction kit. Oxaliplatin-induced p53 translocation into plasma membranous fraction was observed by Western blot analysis (Fig. 13). The protein fractionation was checked with each fraction marker, histone (nuclear fraction), Na^+/K^+-ATPase (membranous fraction) and α-tubulin (cytosolic fraction). To investigate the possible association between lipid raft and oxaliplatin-induced apoptosis, HCC cells were plated in 150 mm dish, after 28 h of oxaliplatin exposure, lipid rafts and non-rafts were separated from the disordered membrane environment using a sucrose density gradient. Total 12 fractions of 1 ml each were collected and analyzed biochemically for their cholesterol and protein contents. Fractions 4 and 5 were identified as lipid rafts, respectively. In the presence of oxaliplatin, p53 and DISC proteins were detected in the lipid rafts region (Fig. 14). We investigated the role of lipid raft which enhanced the aggregation of apoptosis-inducing proteins in response to oxaliplatin. Cells were treated with oxaliplatin in the presence or absence of 2.5-3 mM MBCD for 28 h. Oxaliplatin-induced translocation of p53, Fas, DR5, FADD and caspase-8 into lipid raft was effectively blocked by

MBCD in HepG2 and HCT116 cells (Fig. 15). These results indicate that, lipid rafts are involved in oxaliplatin-induced DISC formation and apoptosis in HepG2 and HCT116 cells.

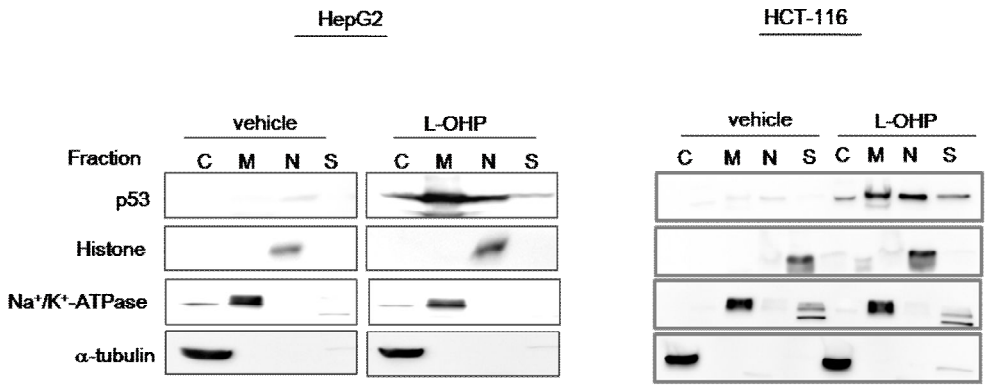


Figure 13. Oxaliplatin induces accumulation of p53 in membranous fraction. HepG2 and HCT116 cells were treated with 100 μM oxaliplatin or untreated (vehicle) for 24 h. Cytosolic protein (C: Cytoplasmic fraction), organelle/membrane protein (M: Membraneous fraction), nuclear protein (N: Nuclear fraction) and cytoskeleton protein (S: Cytoskeletol fraction) were extracted by using proteoExtract subcellular proteome extraction kit. These proteins were used to detect for p53, histone (nuclear marker), Na^+/K^+-ATPase (membrane marker) and α-tubulin (cytosolic marker) by Western blot analysis.

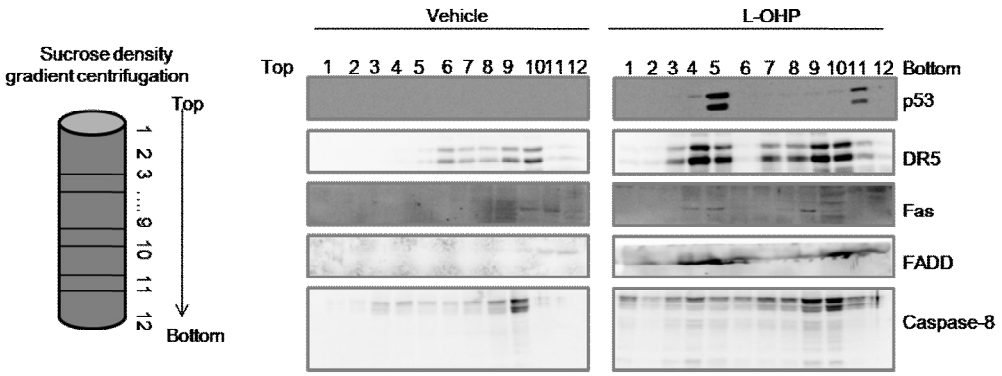


Figure 14. Localization of apoptosis inducing proteins in oxaliplatin induces apoptosis. HepG2 cells were treated with 100 μM oxaliplatin for 28 h. Lipid rafts were separated by using sucrose gradient centrifugation as explained in materials and methods. Total 12 fractions of 1 ml each were collected and analyzed by immunoblotting using antibodies against p53, DR5, Fas, FADD and caspase-8.

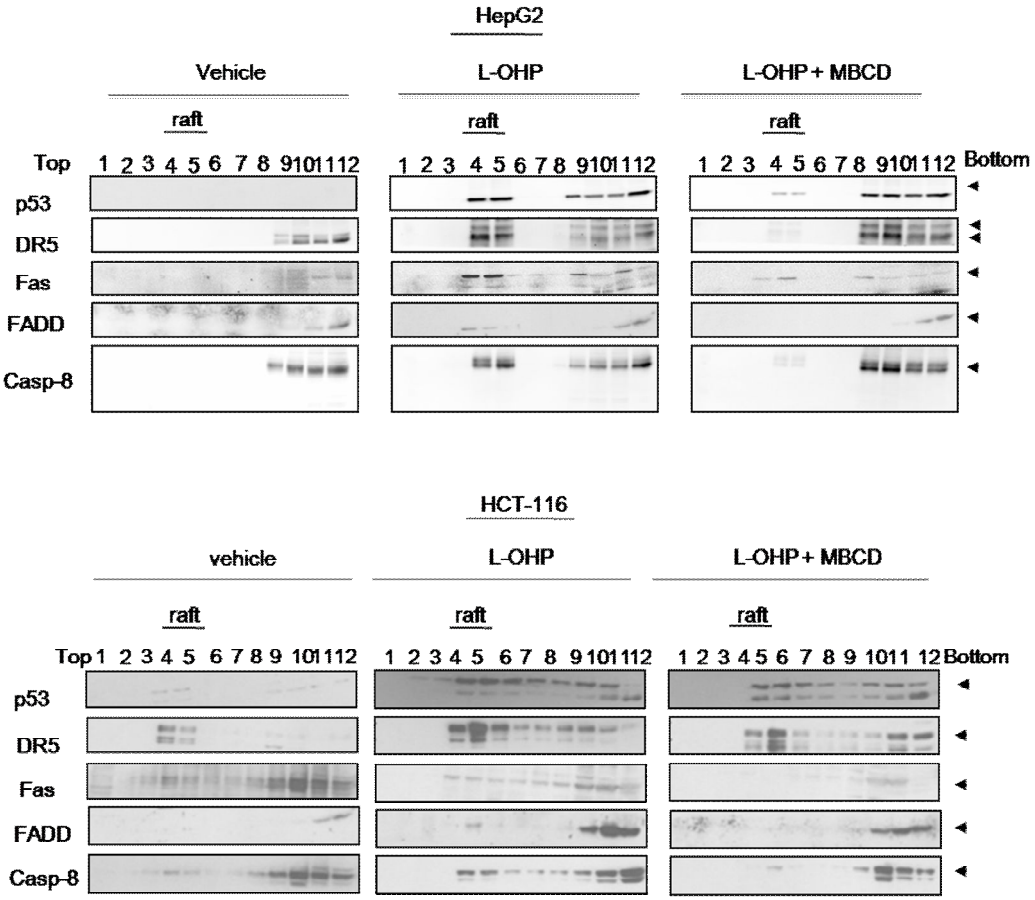
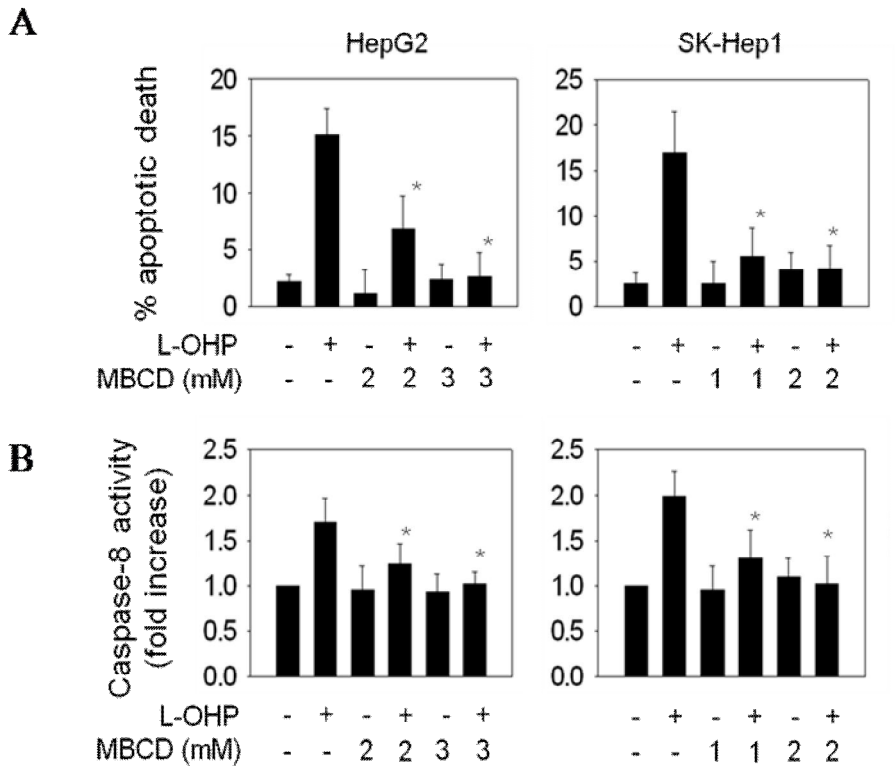


Figure 15. Oxaliplatin-induced translocation of p53 and DISC fractions into the lipid rafts is disrupted by MBCD. HepG2 and HCT116 cells were treated with 100 μM oxaliplatin or 2.5 mM MBCD plus oxaliplatin or untreated (vehicle) for 28 h. Lipid rafts were separated by using sucrose gradient centrifugation. Total 12 fractions of 1 ml each were collected and analyzed by immunoblotting using antibodies against p53, DR5, Fas, FADD and caspase-8.

3.8. Membrane rafts play a critical role(s) in oxaliplatin-induced apoptosis but do not in NF-kB/p53 expression pathway

We examined whether membrane rafts were involved in oxaliplatin-induced apoptosis by the aggregation of DISC proteins in lipid portion. HCC cells were treated with 100 μM oxaliplatin in the presence or absence of 1-3 mM MBCD for 24 h. Apoptotic cell death was observed by hoechst staining in fluorescence microscope. Death cells out of total 500 were calculated. Oxaliplatin-induced apoptosis was highly decreased by the lipid raft disrupting agent MBCD in dose dependent. Treated and untreated cells were subjected to analyze for caspase-8 activation. Oxaliplatin-induced activation of caspase-8 was suppressed with MBCD combination (Fig. 16). For more confirmation, treated cells were subjected to analyze for cleaved caspase-3 by Western blotting. Oxaliplatin-triggered the cleavage of caspase-3 was completely blocked by MBCD. These results indicate that membrane rafts play a critical roles in oxaliplatin-induced apoptosis. In the other aspect, oxaliplatin did not increase the level of death receptors Fas, DR4 and DR5. Next, we examined whether lipid rafts are involved in oxaliplatin-induced NF-kB related p53 expression. 100 μM oxaliplatin was treated in the presence or absence of 1-3 mM MBCD for 24 h, and then cells were subjected to analyze for p53 expression level by Western blotting. Oxaliplatin-induced p53 expression was not affected by MBCD. Next we found that, oxaliplatin-induced p53 expression was involved in NF-kB signaling pathway through the activation of IKKα, which enhanced the degradation of NF-kB inhibiting protein IKB. Here MBCD with oxaliplatin did not suppress the activation of IKKα, which is related for NF-kB activation. To further confirm, cells were treated with oxaliplatin in the presence or absence of MBCD as the function of times (0, 0.5, 1 and 4 h). The NF-kB activation was measured by metridia luciferase activity assay. Oxaliplatin-induced NF-kB activatin was not suppressed by

MBCD combination (Fig. 17). Over all these results confirm that membrane rafts are not involved in oxaliplatin-induced NF-kB/p53 expression pathway.



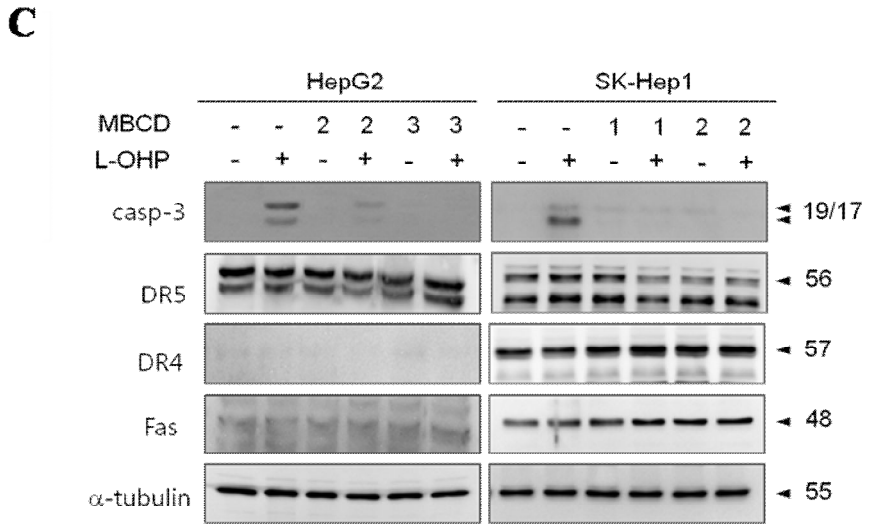


Figure 16. Membrane rafts play a critical roles in oxalipaltin-induced apoptosis. (A) 100 μM oxaliplatin was treated in the presence or absence of 1-3 mM MBCD for 24 h in HepG2 and SK-Hep1 cells (A, B and C). Cells were analyzed for apoptosis by HO/PI staining and apoptotic nuclei were counted under a fluorescence microscope. (B) Caspase-8 activation was assessed by measuring z-IETD-pNA substrate cleavage by FLICE/caspase-8 colorimetric kit. (C) Or cells were subjected to analyzed for cleaved caspase-3, DR5, DR4, Fas and α-tubulin by immunoblotting. *$p < 0.05$.

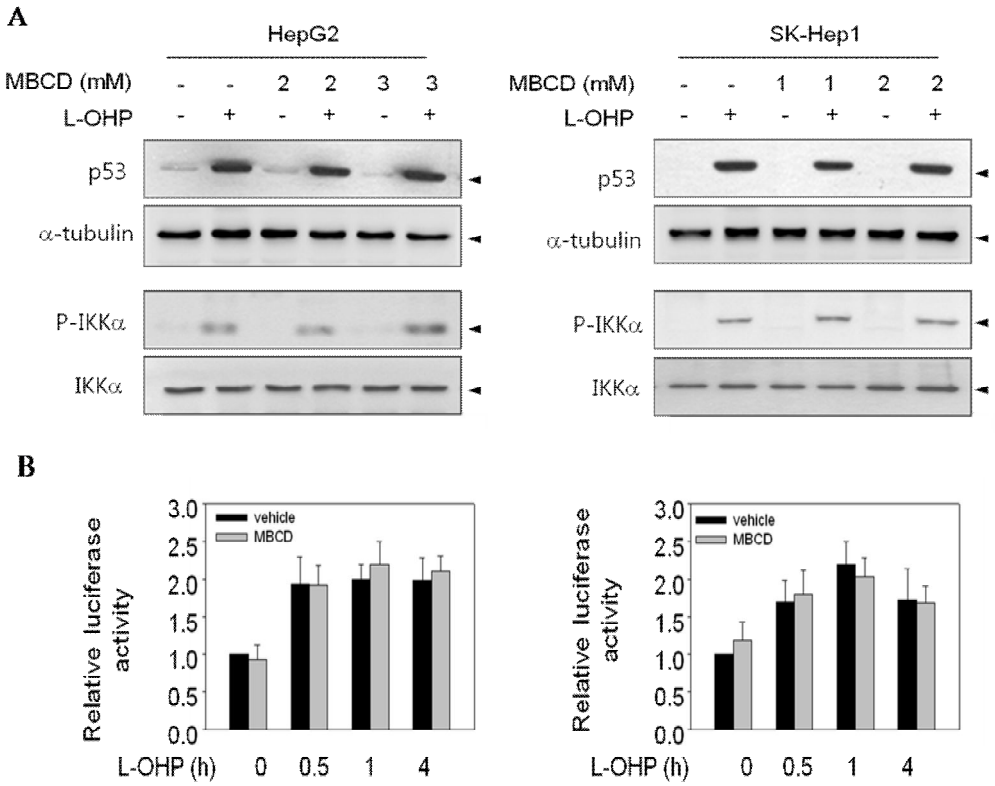


Figure 17. Membrane rafts do not affect on oxaliplatin-induced NF-kB/p53 expression pathway. (A) HepG2 and SK-Hep1 cells were treated with 100 μM oxaliplatin in the presence or absence of 1-3 mM MBCD for 24 h. Total proteins from the cells were collected and analyzed by Western blot for p53, p-IKKα, IKKα and α-tubulin. (B) Cells were treated with 100 μM oxaliplatin in the presence or absence of 2 mM MBCD for various durations (0.5, 1 and 4 h). The amount of metridia luciferase secretion in the medium was assessed by using luciferase substrate and measured in luminometer.

3.9. p53 facilitates the localization of death receptors into lipid rafts and caspase-8-initiated apoptosis

In this result, The DNA-damaging agent oxaliplatin induces p53 protein accumulation in both dose- and time-dependent manners in HepG2 cells through the multiple death receptors and NF-kB signaling pathway. Interestingly the accumulated p53 protein was translocated into lipid rafts in response to oxaliplatin. Here we examine the role of p53 which facilitates the localization of death receptors into lipid rafts and caspase-8-initiated apoptosis. HepG2 cells were transfected with scramble and shRNA p53. After the 48 h of transfection, cells were selected by 1μg/ml of puromycin for 2 weeks. Selected cells were treated by 100 μM oxaliplatin for 28 h. Lipid rafts and non-rafts were separated from the disordered membrane environment using a sucrose density gradient. Total 12 fractions of 1 ml each were collected and analyzed biochemically for their cholesterol and protein contents. Fractions 4 and 5 were identified as lipid rafts. The endogenous p53 was translocated into the lipid rafts fractions in response of oxaliplatin that triggered the DISC formation in the lipid rafts but not in shRNA p53 transfected cell. Elimination of p53 by shRNA reduced the aggregation of death receptors Fas, DR5, adaptor protein FADD as well as caspase-8 in membrane rafts. We examined the transfection efficiency of scramble and shRNA p53 (Fig. 18). These results indicate that p53 facilitates the localization of DISC proteins into the lipid rafts and caspase-8-initiated apoptosis. To further confirm, p53 expression was inhibited by siRNA p53 and then exposed with oxaliplatin for 12 and 24 h. The caspase-8 activation was measured by caspase-8 activity assay, oxaliplatin-induced the level of caspase-8 was decreased by siRNA p53. Or cells were subjected to analyze for caspase-3 activity assay, the activation of caspase-3 was significantly suppressed by p53 interference. The Western blot of cleaved caspase-3 indicated that p53 was

involved in oxaliplatin-induced apoptosis. In order to determine the p53 expression through the NF-kB pathway, siRNA p53 transfected cells were exposed with oxaliplatin for various durations 0.5, 1 and 4 h. Oxaliplatin-induced the acticvation of NF-kB was more increased in the interference of p53 (Fig. 19). This results indicate that p53 is localized in downstream of NF-kB.

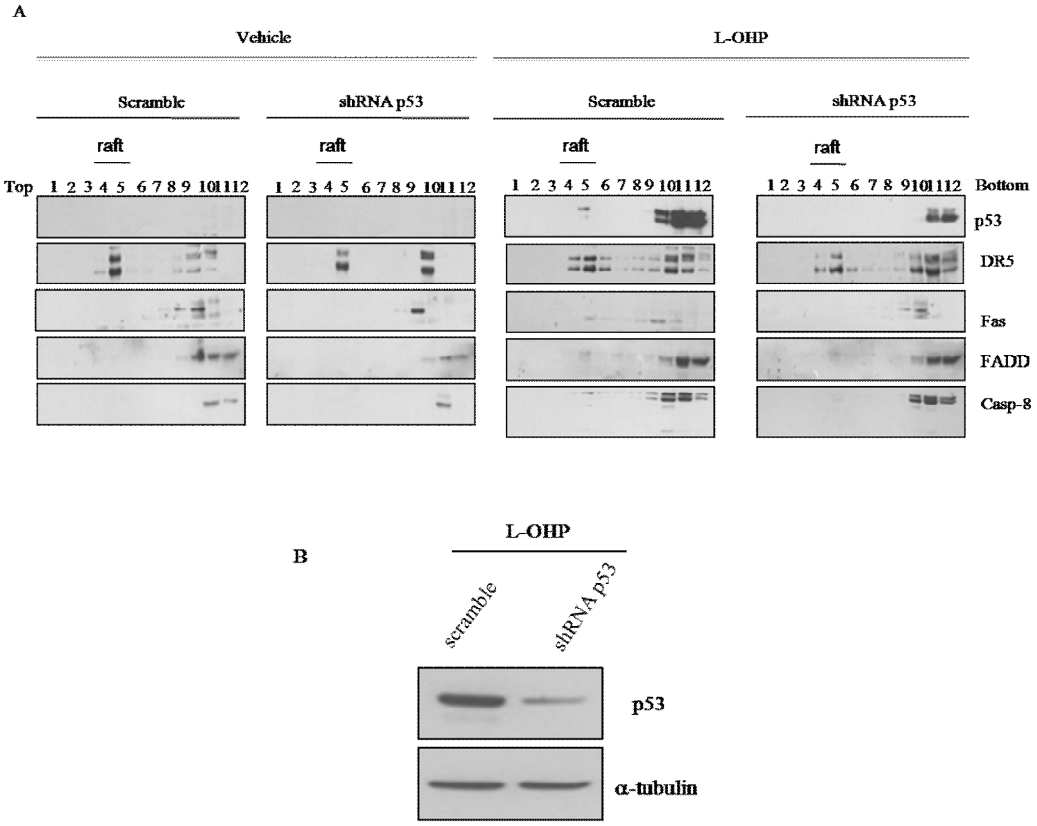
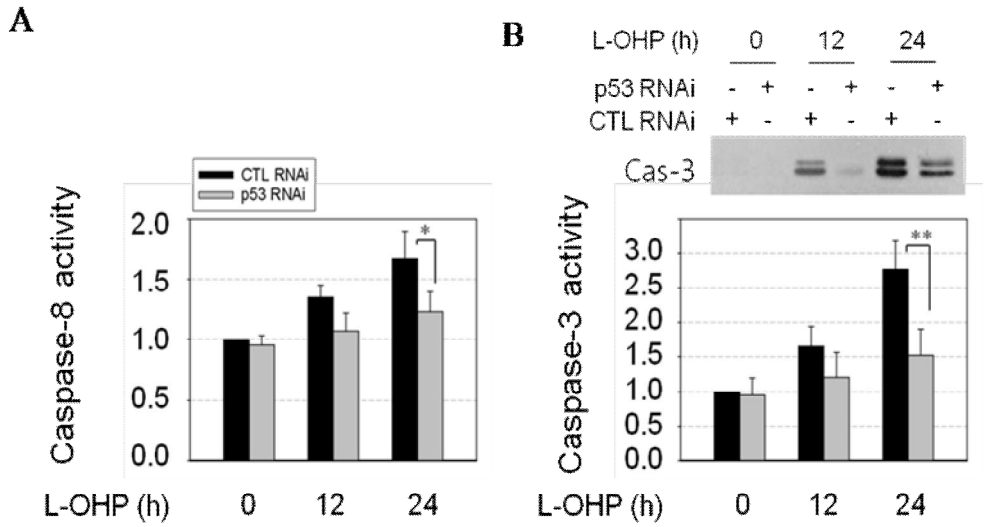


Figure 18. p53 facilitates the localization of death receptors into lipid rafts and caspase-8-initiated apoptosis. (A) HepG2 cells were transfected with shRNA p53 and negative control vectors. After 48 h of transfection, cells were selected by 1 μg/ml puromycin. shRNA p53 selected cells were used for lipid raft isolation. 100 μM oxaliplatin was treated for 28 h and then lipid rafts were separated using sucrose gradient centrifugation as mention in materials and methods. Total 12 fractions of 1 ml each were collected and analyzed by immunoblotting using antibodies against p53, DR5, Fas, FADD and caspase-8. (B) The selection efficiency of shRNA p53 was examined by Western blotting.


A
CTL RNAi
p53 RNAi
Caspase-8 activity
2.0
1.5
1.0
0.5
0.0
*
L-OHP (h)
0
12
24
B
L-OHP (h)
0
12
24
p53 RNAi
CTL RNAi
Cas-3
Caspase-3 activity
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
L-OHP (h)
0
12
24

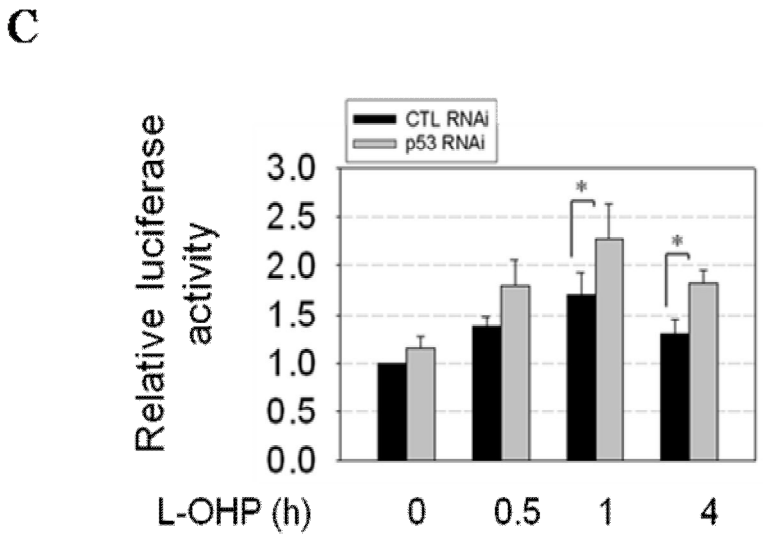

C
CTL RNAi
p53 RNAi
Relative luciferase activity
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
L-OHP (h)
0
0.5
1
4

Figure 19. Role of p53 in caspase-8 and -3 induces apoptosis. (A) HepG2 cells were transiently transfected with control RNAi and p53 RNAi. After 36 h of transfection, cells were treated with 100 μM oxaliplatin for 12 and 24 h (A and B). Caspase-8 activation was performed by measuring IETD-pNA substrate cleavage by FLICE/caspase-8 colorimetric assay kit. (B) Or subjected in Western blot for cleaved caspase-3 or analyzed the caspase-3 activation by measuring DEVD-pNA substrate caspase-3/CCP32 colorimetric assay kit. (C) Transfected cells were treated with 100 μM oxaliplatin for various durations (0.5, 1 and 4 h) and then supernatant was measured for NF-kB activation by luciferase activity assay. *$p < 0.05$, **$p < 0.01$.

3.10. Oxaliplatin-induced p53 interacts with components of lipid raft

Membranes are composed of thousands of different lipid molecules that interact dynamically to form the transient or stable structures used by many proteins as platforms for their activity and for their interactions with other proteins. Usually, transmembrane and peripheral proteins show important specificities in their interactions with lipids. These preferences may be associated either with a defined type of membrane lipid or a given membrane lipid structure. Most proteins are sensitive to their lipid environment, so that their activity can be modified by changes in membrane lipid composition and structure. These changes in membrane lipid composition and structure may have a physiological basis or they may be a response to external stimuli [82]. Here we examined whether p53 interacted with components of lipid rafts in response to oxaliplatin. HepG2 cells were treated with 100 μM oxaliplatin for 24 h, and then collected cells were examined for p53 protein-lipid binding assay as described in materials and methods. Here we tested the sphingostrips and PIP microstrips, which are hydrophobic membranes that have spotted with 15 different biologically active lipids. Expressed p53 was translocated into plasma membrane and interacted with 3-sulfogalactosylceramide, various PtdIns(3), PtdIns(4), PtdIns(5), PtdIns(3, 4)P_2, PtdIns(3, 5)P_2, PtdIns(4, 5)P_2, PtdIns(3, 4, 5)P_2 and Phosphatidic Acid of PIP microstrips in the response of oxaliplatin (Fig. 20). To confirm the p53-lipid binding activity, equal concentration of p53 protein was performed from control and oxaliplatin treated HepG2 cells. Oxaliplatin triggered the p53 protein binding with lipid raft components. Equal concentration of p53 was measured from the treated and untreated cells by Western blot analysis (Fig. 21). The above results indicate that oxaliplatin facilitates p53-binding with components of lipid rafts.

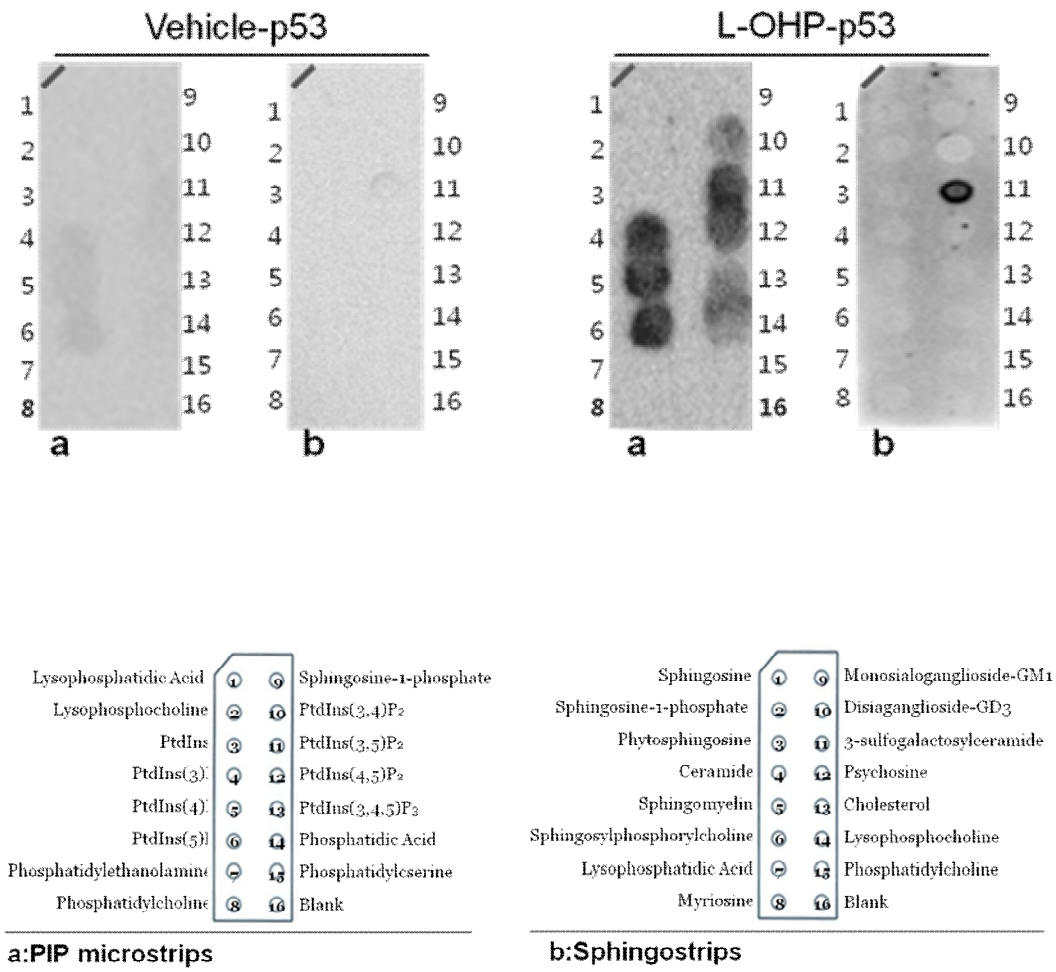


Figure 20. Interaction of p53 with components of lipid raft. HepG2 cells were treated with 100 µM oxaliplatin or untreated (vehicle) for 24 h. 100 µg/ml protein was incubated with the strips (a: PIP microstrips) (b: sphingostrips). p53 antibody was used to detect the p53-lipid binding in treated and untreated cells.

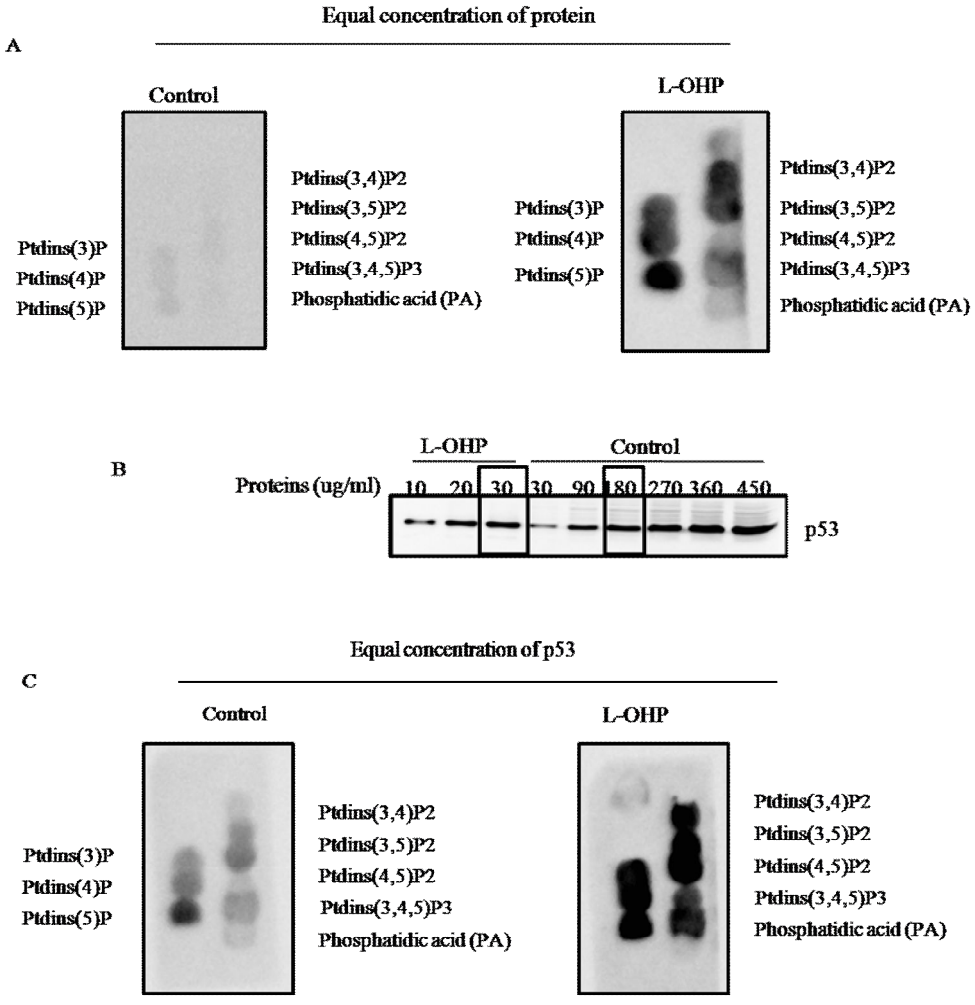

A
Equal concentration of protein
Control
L-OHP
Ptdins(3)P
Ptdins(4)P
Ptdins(5)P
Ptdins(3,4)P2
Ptdins(3,5)P2
Ptdins(4,5)P2
Ptdins(3,4,5)P3
Phosphatidic acid (PA)
B
L-OHP
Control
Proteins (ug/ml)
10 20 30 30 90 180 270 360 450
p53
C
Equal concentration of p53
Control
L-OHP
Ptdins(3)P
Ptdins(4)P
Ptdins(5)P
Ptdins(3,4)P2
Ptdins(3,5)P2
Ptdins(4,5)P2
Ptdins(3,4,5)P3
Phosphatidic acid (PA)

Figure 21. Effect of oxaliplatin on the binding of p53 to various lipid components. 100 μM oxaliplatin was treated or untreated (control) for 24 h in HepG2 cells (A, B and C). (A) After indicated time of oxaliplatin treatment, cells were collected and equal concentration of protein was used for analysing p53 binding with various lipid components. (B) Equal concentration of p53 was determined from the control and oxaliplatin treated cells by immunoblotting. (C) From the Western blot (B) same concentration of p53 protein was used to examine for p53 binding with various PIP components of lipid raft.

3.11. p53 enhances the localization of death receptors into lipid rafts and apoptosis

p53 can regulate the cell death through transcription-dependent and -independent manners. The transcription-independent pathway was examined by the translocation of p53 to lipid rafts. We examined whether p53 enhanced the localization of death receptors into lipid rafts and apoptosis. HepG2 cells were transfected with siRNA control and siRNA p53 and then grow for 36 h in coverslips prior to experiment. After 24 h of oxaliplatin treatment, cells were fixed with 1% formaldehyde and incubated with anti-p53 antibody then coupled by secondary antibody. After washing with 1X-PBS, cells were incubated for 30 min at 4°C with FITC-conjugated CtxB/HO for lipid rafts and nuclear staining. Confocal microscope was performed to determine the localization of p53 and DISC proteins. Oxaliplatin induced the translocation of p53 into the lipid rafts, which enhanced the aggregation of death receptors and other apoptosis inducing proteins (Fig. 22). In the inhibition of p53 by siRNA eliminated the oxaliplatin-induced localization of death receptors into lipid rafts and apoptosis. These results indicate that p53 is involved in oxaliplatin-induced DISC localization into the lipid rafts and apoptosis in HepG2 cells.

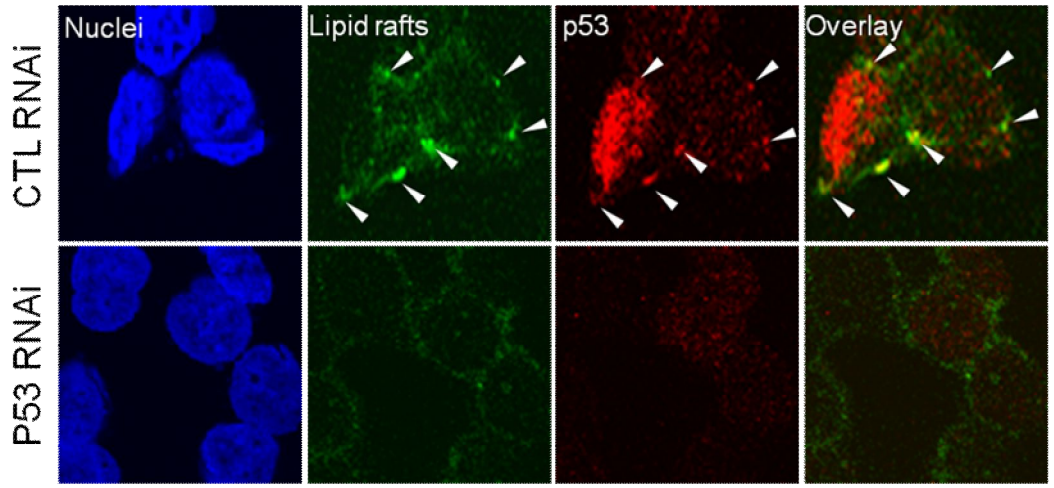


Figure 22. p53 enhances the localization of death receptors into lipid rafts and apoptosis. siRNA control and siRNA p53 transfected HepG2 cells were grown in coverslips overnight and treated with 100 μM oxaliplatin for 24 h. After fixation, cells were stained with anti-p53/FITC-CTxB/HO and observed under a confocal microscope.

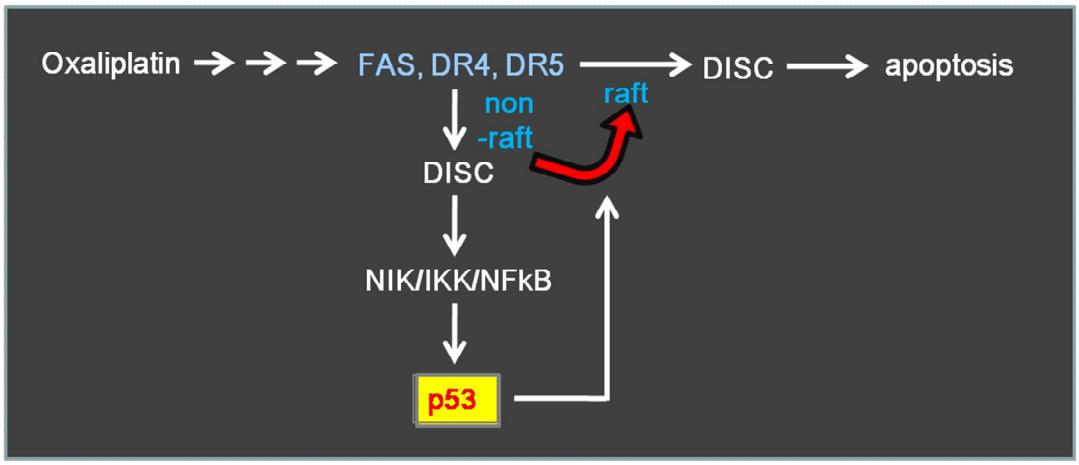


Figure 23. A positive feedback regulation between p53 and death receptor/caspase-8-initiated apoptotic pathway in oxaliplatin-induced apoptosis.

CHAPTER 4: DISCUSSION

In this study, we found that apoptosis-induced by DNA-damaging agent such as oxaliplatin was regulated by the p53. However, the reduction of p53 expression by siRNA p53 suppressed the oxaliplatin-induced apoptosis in HCC cells. We also investigated the effects of oxaliplatin on p53 protein and mRNA expression in SK-Hep1 and HepG2 cells. Furthermore, we examined if the p53 expression would be regulated by proteosomal protein degradation pathway. The inhibition of MDM2 expression by siRNA MDM2 or inhibitor MG132 did not suppress the p53 expression level in response to oxaliplatin. Instead of that, p53 expression level was suppressed by CHX combination with oxaliplatin. Several reports have suggested that translational regulation may also contribute to p53 induction after DNA damage. p53 is a critical cellular protein that plays an essential role in regulating the cell cycle and the cellular response to certain environmental or genotoxic stresses. Under normal growth conditions, p53 is a short-lived protein and is expressed at relatively low basal levels within the cell. In response to various cellular stresses, including exposure to DNA-damaging agents, UV and γ irradiation, hypoxia, and nucleotide depletion, p53 is rapidly induced and results in a transciptional-dependent and –independent apoptosis [31]. However, another study shows that the reduction of MDM2 expression does not alter the response of wild-type p53 to either 5-FU or doxorubicin treatment [83]. In the initial reports of p53 induction after ionizing irradiation, the protein-synthesis inhibitor CHX was shown to block p53 induction, and marked increases in labeling of p53 protein after treatment [32, 33]. With compare these reports, p53 is involved in oxaliplatin-induced apoptosis. It is also known that oxaliplatin-induced p53 expression is regulated by protein synthesis pathway but not proteosomal protein degradation.

In addition, we would like to investigate if multiple death receptors are involved in

oxaliplatin-induced p53 expression and apoptosis. In our previous study, oxaliplatin induced ROS production which in turn contributed in p53 expression. Oxaliplatin-induced p53 expression was completely suppressed by ROS scavengers i.e. NAC, BHA and tiron. In this study, oxaliplatin-induced p53 expression is regulated by multiple death receptors. The DNA-damaging agent, oxaliplatin related ROS production may be involve in death receptors activation and p53 expression. A recent report has shown that the induction of apoptosis by DNA-damaging agents was mediated by ROS-dependent clustering of death receptor. Cisplatin promotes ROS production, which in turn contributes to Fas receptor aggregation and cell death. The novel coupling between ROS and Fas clustering likely plays a significant role in apoptosis triggered by DNA-damaging agents in Fas-expressing leukemia cells [84].

Here, we would like to know if the NF-kB involves in a p53 transcriptionally expression. For this, NF-kB activation was measured by the treatment of oxaliplatin as the function of times. Oxaliplatin induced NF-kB activation through the MAP4K4 and MAP4K14 that activated the IKKα and degradated the inhibitory IKB proteins. In the inhibition of NF-kB inducing kinase by siRNA NIK significantly suppressed the p53 transcription level. It is reported that NF-kB shares a Rel RHD, which mediates DNA binding, dimerization, and interactions with specific inhibitory factors, the IKB, which retain NF-kB dimers in the cytoplasm [46]. Many stimuli activate the NF-kB, mostly through IKK-dependent phosphorylation and subsequent degradation of IKB proteins. The liberated NF-kB dimer enters into the nucleus, where they regulate transcription of diverse genes encoding cytokines, growth factors, cell adhesion molecules, and pro- and antiapoptotic proteins [47]. NF-kB is activated by a variety of signals through mechanisms that result in phosphorylation and degradation of the IKB proteins, the best understood being activation of IKK in response to TNFα signaling [48]. The NF-kB signaling pathway is regulated by the cytosolic-nuclear shuttling and modulation of the

transcriptional activity [46]. These results support our consideration.

Our results indicate that NIK is involved in activation of NF-kB which finally regulates the transcriptional expression of p53 through the signal of multiple death receptors such as Fas, DR4 and DR5. CD95/Fas and TRAIL R1/R2 DISC consists of oligomerized, in which receptors DD-containing adaptor molecule FADD, Rip1, procaspase-8, and procaspase-10 [85]. This is then released into the cytosol to propagate the signal in downstream mediator of NIK and IKK activation. Signal through adaptor molecules and kinases to activate the IKKα kinases, which regulate the proteolysis of IKBα and enable NF-kB heterodimers to translocate to the nucleus and activate transcription [86]. Recruitment of caspase-8 and -10 in the DISC, which is assembled upon triggering of several death receptors, such as TNF receptor 1, Fas and TRAIL, induces pro-caspase dimerization that is followed by proteolytic cleavage [74]. Caspase-8, and -10 have been shown to co-immunoprecipitate with RIP1, NIK, and IKKs upon overexpression in HEK293T cells [75] providing a possible mechanistic link. *In vitro* pull-down assays with recombinant proteins showed a direct interaction with RIP1 and NIK , whereas the binding of IKKs is an indirect effect [78]. Our results represent the caspase-8 catalytic activity in signal transduction through NIK, IKK and IKB contribute to NF-kB activation, but NF-kB activation after NIK and IKK signaling might be independent of caspase-8 enzymatic activity and involves in a scaffolding function of caspase-8 and -10.

Usually, FasL and TRAIL allow the activation of Fas and TRAIL receptors in the initiation of signal transduction. We have observed the role of this ligand in p53 expression and apoptosis with oxaliplatin combination in HCC cells. There was no any significant change in the presence of FasL and TRAIL in cell death. When compared with nontransformed cells, cancer cells are more sensitive to TRAIL-induced apoptosis following exposure to TRAIL treatment. Selective sensitization of tumor cells to TRAIL-induced apoptosis can often be attributed to greater

expression of the TRAIL receptor [87]. TRAIL induces apoptosis in a variety of cancer cell lines regardless of p53 status, therefore it might be a useful therapeutic strategy, particularly in cells in which the p53-response pathway has been inactivated, thus helping to circumvent resistance to chemo- and radiotherapy [87, 88]. Although one of the attractive features of TRAIL is its ability to kill cancers with mutations in the p53 gene, the combination of TRAIL with chemotherapeutic agents has been found to be particularly effective in killing cancers with wild-type p53 [87, 88]. However, in this study, FasL and TRAIL does not show the synergistic effect with oxaliplatin-induced p53 expression and apoptosis in wild-type p53-harboring HCC cells.

In this study, p53 is translocated into lipid rafts and enhances the aggregation of death receptors such as Fas and DR5, adaptor molecules FADD form DISCs that trigger apoptosis in response to oxaliplatin. Indeed, we have observed that expressed p53 strongly facilitated the translocation of death receptors and adaptor proteins into the membrane rafts, which was disrupted by MBCD. This was confirmed by shRNA p53 which prevented the DISC formation and apoptosis. Among the different receptors that bind to Fas and TRAIL, only two, DR4 and DR5, contain a cytoplasmic death domain and trigger apoptosis, whereas the others (TRAIL-R3, TRAIL-R4 and osteoprotegerin) are decoy receptors. Upon activation, DRs form DISC that consists of receptors, FADD and initiator caspase-8, resulting in activation of downstream caspases, with or without mitochondrial amplification of the signal [89]. Functional activity of DRs, such as Fas and DR5, requires their physical association with lipid rafts, which serve as plasma membrane platforms for DR-initiated signals in formation of efficient DISCs [90, 91]. Lipid rafts are liquid-ordered membrane microdomains with a unique protein and lipid composition found on the plasma membrane of mammalian cells. A large number of signaling molecules are concentrated within rafts, which have been proposed to function as signaling

centers capable of facilitating efficient and specific signal transduction [92]. Differential localization of signaling molecules to lipid rafts and the bulk plasma membrane could control the access of signaling molecules to each other. The raft microenvironment is also capable of altering the behavior of signaling proteins [93]. Lipid rafts play a role in clustering or aggregating surface receptors and adaptor molecules into membrane complexes at specific sites and are shown to be essential for initiating signaling from a number of receptors, particularly in the initiation of DR5 and Fas-mediated apoptosis during chemotherapy [94]. The availability of caveolin-1 as a marker protein allowed the development of biochemical techniques for the isolation of specialized membrane domains that purified with caveolin-1. The caveolin-associated membrane fraction was characterized by a low buoyant density in sucrose density gradients [95]. Flotillin-1 and flotillin-2 have distinct cell-specific expression patterns and can form stable hetero-oligomeric complexes with caveolins when co-expressed in the same cell [96]. Lipid raft disruption by MBCD from the plasma membrane has been reported to deregulate a number of intracellular signaling pathways and cross-talk between different receptor systems, indicating that raft integrity is critical for intracellular signaling triggered by cell surface receptors [60]. Apoptosis in mammalian cells is modulated by extrinsic and intrinsic signaling pathways through the formation of death receptor-mediated DISC and mitochondrial-derived apoptosome, respectively [97]. If we compare our findings with this report, the lipid raft depleting agent MBCD blocked the localization of death receptors together with DISC proteins into the membrane rafts in the 4 and 5 fractions including caveolin-1 in response to oxaliplatin in HCC and HCT116 cells. But the integrity of lipid raft is not involved in NF-kB-mediated p53 expression through the NIK/IKK signaling

Our findings reported here, the p53 recruited with the components of lipid rafts as a major mechanism in oxaliplatin treatment, which enhanced the aggregation of DISC proteins into the

lipid rafts in HCC cells. We have found the p53 interaction with various phospholipid and sphingolipid components of plasma membrane such as PtdIns(3)P, PtdIns(4)P, PtdIns(5)P, as well as with their different phosphorylated on the inositol ring including PtdIns(3,4)P_2, PtdIns(3,5)P_2, PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 due to oxaliplatin treatment. Inositol phospholipids are focused in plasma membrane trafficking events. Reversible phosphorylation of phosphatidylinositol generates spatially localized signals on membranes that recruit or activate proteins essential for cell membrane budding, fission and fusion [98]. Phosphatidylinositol (3,4,5) triphosphate (PI[3,4,5]P_3), phosphatidylinositol (4,5)-biphosphate (PI[4,5]P_2) is now recognized as an important plasma membrane signal that establishes sites for vesicular trafficking and membrane movement. The signaling role of PI(4, 5)P2 is mediated through interactions with proteins required for membrane trafficking that contains PI(4, 5)P2-binding domains [99]. These lipids bind tightly to proteins at focal adhesion sites where they are thought to activate these proteins to assemble at the contact site [100]. Binding proteins are most commonly affected by PIP_2, but other inositol lipids such as PI(3,4)P_2 and PI(3,4,5)P_3 also affect several actin binding proteins [101]. Caveolae and detergent-insoluble, glycosphingolipid-enriched domains (DIGs) are cholesterol-enriched membrane domains that have been implicated in signal transduction because a variety of signaling proteins as well as PtdInsP_2 are compartmentalized in these domains [102]. Common themes of localized signal generation and the spatially localized recruitment of effecter proteins appear to underlie mechanisms employed in signal transduction and membrane trafficking events [103]. Although most of the work connecting p53 and sphingolipids has focused on ceramide, considerable evidence relates p53 and more complex members of the sphingolipid family [104]. Our data are supported by this report, which indicates that oxaliplatin induces the binding of p53 proteins with various components of lipid rafts and enhances the plasma membrane trafficking for

signal transduction. In this study, we observed the oxaliplatin-induced expression of p53 protein as well as mRNA level. Usually, p53 is expressed through the protein synthesis or proteosomal protein degradation pathway in response to DNA-damaging agents. Here we found that multiple death receptors are involved in oxaliplatin-induced p53 expression through the NIK/IKK/NF-kB signaling pathway. Accumulated p53 translocates into the lipid rafts and finally facilitates the DISC formation and apoptosis.

CHAPTER 5: CONCLUSION

Oxaliplatin induced p53 accumulation in various cancer cells such as HepG2, SK-Hep1, A549 and HCT116. Multiple death receptors including Fas, DR4 and DR5 involved in p53 transcription in response to oxaliplatin in HCC cells, which induced apoptosis. Moreover, adaptor protein FADD and Rip1 required to increase p53 expression. However, oxaliplatin-induced p53 induction was not suppressed by lipid raft disrupting agent methyl-beta cyclodextrin. Therefore, p53 expression preferentially localized in non-raft region and link to NF-kB activating machinery instead of raft region. Finally, the p53 protein was translocated to plasma membrane by oxaliplatin and enhanced membrane rafts-engaged death receptor activation. These results demonstrate that p53-mediated raft formation and DISC protein recruitment are involved in initiation of apoptosis in response to oxaliplatin.

CHAPTER 6: ABSTRACT

Molecular mechanisms of oxaliplatin-induced p53 expression and apoptosis

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Oxaliplatin is a platinum based chemotherapeutic drug in the same family of cisplatin and carboplatin. In the present study, we found the novel role of tumor suppressor protein p53 in oxaliplatin-induced apoptosis through aggregation of death inducing signaling complex (DISC) in lipid rafts in hepatocellular carcinoma (HCC) and HCT116 cells. p53 levels were increased through the death receptors such as Fas, DR4 and DR5, and when the inhibition of the expression of these by siRNA, the p53 protein and mRNA levels were highly suppressed in response to oxaliplatin. Generally, p53 expression is regulated by the oncoprotein mouse double minute 2 (MDM2) through the ubiquitination and proteosomal protein degradation. However in HepG2 and SK-Hep1 cells, the proteosome inhibitor MG132 or siRNA MDM2 did not regulate the p53 induction.

Death receptors (Fas, DR4, and DR5) dependent NF-kB pathway was involved in oxaliplatin-induced p53 induction. Firstly, NF-kB was activated by death receptors, adaptor molecules FADD, RIP1 form DISC that initiated the nuclear factor inducing kinase (NIK) including MAP4K4 and MAP3K14. The activation of inhibitor of kappa B kinase (IKK) enhanced the degradation of inhibitor of kappa B (IKB), and then activated NF-kB was translocated into nucleus, and transcriptionally expressed the p53. Furthermore, p53 played

a key role in apoptosis by regulating the death receptors into the lipid rafts. p53 was induced by DNA damaging agent oxaliplatin, and was finally localized to plasma membrane, particularly in raft regions. In addition, p53 was redistributed into large and highly buoyant lipid raft structures in HepG2 as well as HCT116 cells that underwent apoptosis in response to oxaliplatin. Furthermore, p53 interacted with various lipid components such as 3-sulfogalactosylceramide and phosphoinositide (PIP). Methyl-beta-cyclodextrin (MBCD), a raft disrupting agent, suppressed localization of p53 and DISC proteins in lipid raft and blocked oxaliplatin-induced apoptosis. However, MBCD did not prevent oxaliplatin-induced p53 expression, suggesting that, the death receptors regulating p53 expression may be localize in non-raft region. Inhibition of p53 with shRNA p53 prevented raft formation and localization of DISC protein and apoptosis. p53 enhanced the localization of death receptors into lipid rafts and caspase-8-initiated apoptosis which was detected by immunofluorescence technique. In conclusion, oxaliplatin induced p53 by transcriptional mechanism through death receptors-dependent NIK/IKK/IKB/NF-kB activation pathway. Furthermore, p53 was translocated to membrane by oxaliplatin and enhanced membrane rafts-engaged death receptor, caspase-8 and-3 activation. These results demonstrate that p53-mediated raft formation and DISC protein recruitment are involved in initiation of apoptosis in response to oxaliplatin.

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