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REVIEW（JAOB/Rising Members Award）

Defect in Ser46 Phosphorylation of p53 Protein：A Resistance Mechanism against p53 Gene Transfer in Oral Squamous Cell Carcinoma Cells

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Abstract：Oral squamous cell carcinoma (SCC) shows frequent metastasis and recurrence, ultimately with a poor outcome. The long-term survival rates of patients with oral SCC have not significantly been improved. The *p53* tumor suppressor gene is known to be one of the most commonly mutated genes in human cancers, including oral SCC. *p53* gene replacement therapy to treat such cancers has become an intensive area of research. However, the introduction of wild-type p53 protein is unable to induce apoptosis in all tumor cases, at least in part, due to their resistance to exogenous *p53*. Recently, we reported that a defect in the phosphorylation of p53 protein at serine 46, which is critical for p53-mediated apoptosis, is responsible for the acquisition of resistance to p53 gene transfer in oral SCC cells. In this review, we focus on the regulation of Ser46 phosphorylation and discuss the contribution of its dysregulation to resistant mechanisms against p53 gene transfer in oral SCC.

Introduction

The p53 tumor suppressor protein was identified more than 20 years ago as a cellular protein that interacts with a viral oncoprotein, simian virus 40 (SV40) large T antigen. The p53 cDNA isolated from tumor cells (*i. e.*, Mutant p53) exhibits oncogenic activity, and was therefore initially recognized as an oncogene[1)]. Nevertheless, identification of the wild-type p53 gene and subsequent functional studies in the late 1980's uncovered its real action as a tumor suppressor gene[2)]. Since then, as many as fifty-thousand papers have been published, demonstrating that p53 mutation occurs at a high frequency in diverse types of human cancer, which has been confirmed through analyses of more than 2,500 tumors and tumor cell lines[3,4)].

p53 plays a critical role in the cellular response to genotoxic stress as a major defense against cancer, maintaining genome integrity following genotoxic stress to prevent cells from undergoing inappropriate growth and division. p53 regulates a wide range of target genes responsible for different cellular outcomes, such as cell cycle arrest, senescence, or apoptotic cell death. Among them, apoptosis is an evolutionarily conserved process through which organisms remove abnormal cells, and thus represents a fundamental roadblock to tumorigenesis[5—7)]. Therefore, loss of the

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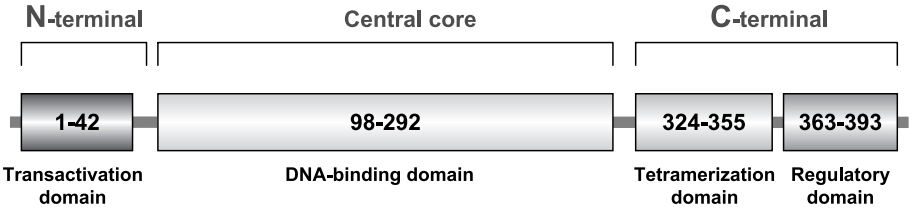

Fig. 1 Structure of human p53

Values in the shaded boxes indicate numbers of/positions of amino acids (aa). p53 protein consists of four functional domains : the transactivation domain is located in the N-terminal, DNA-binding domain is located in the central core, and the tetramerization and regulatory domains are located in the C-terminal.

p53-dependent apoptosis caused by p53 mutation is believed to be a critical step in the carcinogenesis of many types of human malignancy[8—10].

In view of the impact of apoptosis on therapy outcomes, strategies to restore apoptotic p53 pathways in tumor cells have been intensively pursued in recent years. The restoration of wild-type p53 (wt-p53) using adenoviral vectors could be a potential therapeutic approach, and is already undergoing clinical evaluation in patients with various types of human tumor. However, the introduction of wt-p53 causes different responses to tumors, some of which are resistant to p53-mediated apoptosis[11], and the factors that influence the efficacy of p53 gene transfer remain to be investigated.

Phosphorylation of the N-terminal serines of the p53 protein contributes to its stabilization and activation. In particular, many lines of evidence have demonstrated the importance of Ser46 phosphorylation for the apoptotic function of p53. We have reported that a defect in Ser46 phosphorylation is responsible for the acquisition of p53 resistance in an oral squamous cell carcinoma (SCC) cell line[12]. This brief review focuses on the regulation of Ser46 phosphorylation, and discusses the contribution of its dysregulation to a resistance mechanism against p53 gene transfer in oral SCC.

The Structure and Regulation of p53 Protein

p53 acts as a transcription factor, with a mediating effect on the expression of its own downstream target genes[13,14]. The structure and sequence of the gene corresponds to key features of the protein and are well-conserved in all vertebrates[15]. The p53 protein consists of four functional domains : an N-terminal transactivation domain, central DNA-binding domain, tetramerization domain, and C-terminal regulatory domain (Fig. 1). Under normal conditions, the amount of cellular p53 protein is maintained at very low levels, which is tightly controlled by mouse double minute 2 (Mdm2), one of the main targets and a negative regulator of p53. Mdm2 binds and inactivates p53 by both repressing its transcriptional activity and promoting its ubiquitinization and proteasomal degradation[16—19].

Numerous studies have demonstrated that the stabilization and activation of the p53 protein are regulated by posttranslational modifications and its interaction with other proteins. p53 is phosphorylated at a number of serine residues in the N- and C-terminal domains[20,21]. In particular, the phosphorylation of N-terminal serines, including Ser6, Ser9, Ser15, Ser20, and Ser37, inhibits its ability to bind to Mdm2[22—29]. In response to various stresses such as DNA damage (ultraviolet light, ionizing radiation, and genotoxic drugs), hypoxia, oncogene overexpression, or viral infection, the ability of Mdm2 to alter p53 expression is diminished, resulting in stabilization of the p53 protein. Mdm2 is also targeted and degraded by p14ARF, causing p53 release from the p53-Mdm2 complex in the nucleus[30]. The release of p53 due to Mdm2-mediated inhibition leads to stabilization of the p53 protein and the activation of its transcriptional activity, as a consequence of which p53 modulates the expression of its downstream target genes

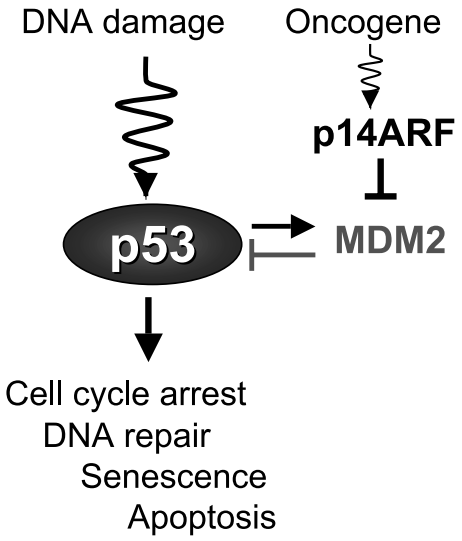

Fig. 2 Model of cell cycle regulation by p53
p53 expression is tightly regulated by mouse double minute 2 (MDM2) in a negative feedback loop, whereas MDM2 expression is controlled by p14ARF. Genotoxic stresses as well as oncogene activation disrupt the MDM2-p53 interaction that leads to p53 stabilization, thus modulating the activation of its downstream genes involved in cell cycle arrest, DNA repair, senescence, or apoptosis.

involved in cell cycle arrest, DNA repair, senescence, apoptosis, and the inhibition of angiogenesis and metastasis[9,31,32] (Fig. 2).

Role of Ser46 Phosphorylation in p53-mediated Apoptosis

p53 triggers apoptosis when cells suffer severe, irreparable damage, whereas it causes cell-cycle arrest when the damage is mild, thereby enabling the cell to repair the damage[33,34]. Depending on the level of DNA damage, p53 preferentially modulates the transcription of either pro-arrest or pro-apoptotic target genes. Upon severe DNA damage, p53 activates the expression of multiple target genes whose products cause apoptosis, although evidence indicates that p53 also induces apoptosis in a transcription-independent manner[13,35]. p53 serves as a regulator of the apoptotic process that can modulate key control

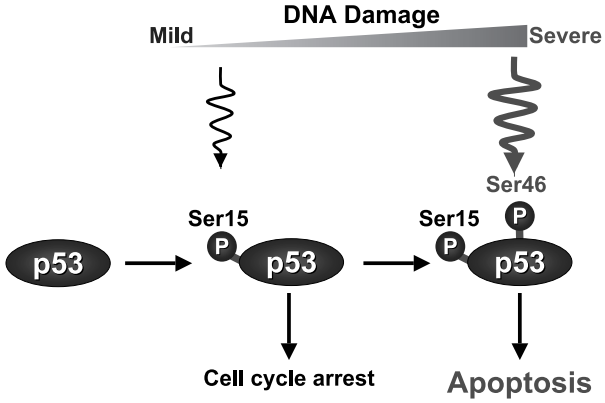

Fig. 3 Ser46 phosphorylation in p53 mediates apoptosis induced by severe DNA damage
Upon "mild" DNA damage, Ser15 and some other serine residues are phosphorylated, thereby promoting cell cycle arrest. In contrast, severe and irreparable damage induces phosphorylation at Ser46, which irreversibly drives cells toward apoptosis.

points in both the extrinsic (consists of cell surface receptors) and intrinsic pathways (centers in mitochondria)[36]. Therefore, p53 apoptotic target genes are involved according to their functions in death receptor pathways (Fas DR4, DR5/KILLER, DcR1, DcR2) and mitochondrial machinery (Bax, PUMA, Noxa, Bid, Apaf1, p53AIP1, PERP, PIDD, Caspase-6), and others (p53DINP1, GML, STAG1, p53CABC1, p53RDL1) play distinct roles in p53-mediated apoptosis[7,37]. The activation of promoters involved in apoptosis is at least partially determined by the overall level of p53 protein, posttranslational modifications of p53 including phosphorylation, such as Ser46, acetylation and methylation, and interaction with proteins, such as Mdm2 or other p53 family members[38].

The phosphorylation of Ser46 following severe DNA damage has been shown to be critical for inducing p53-mediated apoptosis[39]. Severe, irreparable DNA damage induces phosphorylation at Ser46, and Ser46-phosphorylated p53 selectively transactivates pro-apoptotic genes, including *p53AIP1*, which is critical for p53-mediated apoptotic induction. Ser46 phosphorylation changes the affinity of p53 to its target

gene promoters, shifting from pro-arrest to pro-apoptotic genes[39-41] (Fig. 3). Therefore, Ser46 phosphorylation is considered to be a "point of no return", because it irreversibly drives cells toward apoptosis[39].

Phosphorylation of Ser46 has been shown to be mediated by several protein kinases such as p38-mitogen-activated protein kinase (p38-MAPK)[42], homeodomain-interacting protein kinase-2 (HIPK-2)[43,44], protein kinase C δ (PKCδ)[45], and dual-specificity tyrosinephosphorylation-regulated kinase 2 (DYRK2)[46]. p38-MAPK phosphorylated p53 at Ser33 and Ser46. Mutation of these sites decreased p53-mediated and UV-induced apoptosis[42]. A novel pro-apoptotic kinase, protein kinase C δ (PKCδ), is also involved in the phosphorylation of p53 on Ser46. PKCδ-mediated phosphorylation is required for the interaction of PKCδ with p53 to induce apoptotic cell death in the cellular response to DNA damage[45]. Coincidently, p53DINP1, a p53 pro-apoptotic target gene which also plays a role as a cofactor in the phosphorylation of Ser46[47], associates with PKCδ upon exposure to genotoxic agents. HIPK2 is activated by severe or prolonged genotoxic stress, and selectively phosphorylates Ser46 on p53[43,44,48]. HIPK2 inhibits nuclear export and degradation mediated by Mdm2, thereby promoting the transcriptional activity and apoptotic function of p53[49]. A recent study demonstrated that, under mild DNA damage, Mdm2 induced by p53 ubiquitinates HIPK2 and targets it for degradation, which prevents Ser46 phosphorylation and induces growth arrest instead of apoptosis. In contrast, the reduction of Mdm2 levels caused by severe DNA damage results in the stabilization of HIPK2, leading to the phosphorylation of Ser46[48]. The most recent identified kinase, DYRK2, is reported to directly phosphorylate p53 at Ser46[46]. Upon exposure to genotoxic stress, DYRK2 translocates into the nucleus, leading to Ser46 phosphorylation. Consistently, overexpression of DYRK2 induces p53AIP1 expression and apoptosis in a Ser46 phosphorylation-dependent manner.

p53 Gene Therapy and Oral Cancer

Oral SCC is the most commonly occurring malignant tumor of the oral cavity, with a poor prognosis, resulting in major morbidity and mortality[50]. The frequency of p53 mutations in oral SCC is quite high[51], and p53 mutation is the most prominent genetic alteration in oral cancer[52]. Modifications of conventional cancer therapies, including surgery, radiotherapy, and chemotherapy, have not improved the survival rates of patients with oral cancer.

Gene therapy can be defined as the treatment of a disease by replacing, altering, or supplementing a gene whose absence or abnormality is responsible for it[53]. Since most human oral SCCs harbor a mutated p53 gene, the replacement of the mutated gene with wt-p53 has been considered as a potential therapeutic approach to treatment. Recombinant adenovirus expressing wt-p53 (Ad-p53) is one of the promising therapeutic agents for the treatment of oral cancer[54,55].

The introduction of Ad-p53 either as a single agent or combined with other agents efficiently induced apoptosis in some cancer cells, including oral and head-neck cancer[56—61]. Furthermore, it has been shown that tumor cells transduced with the wt-p53 gene can inhibit the growth of adjacent non-transduced tumor cells *in vivo*, which is possibly due to an anti-angiogenic function of p53[62]. In addition to p53, the two p53 family members, p73 and p63, might be useful to broaden cancer therapy research[63,64]. These proteins share similar transcriptional functions and the ability to induce apoptosis, although they are rarely mutated in human cancers, and each of them appears to play a distinct role in development and tumor suppression[65,66]. Several lines of evidence suggest that p73 can induce tumor cell apoptosis in a p53-dependent and -independent manner[63,64].

While the transfer of wt-p53 efficiently induces apoptosis in some cancer cells (p53-sensitive cells), it does not do so in others (p53-resistant cancers)[11,67]. Numerous studies have reported that certain forms of mutant p53 protein expressed in p53-resistant tumors interfere with the action of wt-p53, which have a gain of function or a dominant negative function by forming a heterotetramer complex with wt-p53, thereby inhibiting wild-type functions[13]. Such mutations are observed in approximately 74% of p53-

mutated human cancers and exhibit a selective advantage in carcinogenesis[68—70]. Furthermore, previous studies have shown that the common polymorphic forms of the p53 at codon 72 influence the gain-of-function properties of mutated p53[71,72]. These polymorphic alleles encode either proline (72P) or arginine (72R), exhibiting significant differences in the biochemical properties of the p53 protein. In SCC cells, mutant p53 proteins with 72R are found more commonly than those with 72P[73,74]. Specific p53 mutants containing the 72R allele have been shown to interact with p73, and repress p73-mediated apoptosis in cancer cells[73,75,76]. Recently, we showed that inhibition of the mutant p53 protein containing 72R by mutant-specific, small interfering RNA (siRNA) simultaneously restored p53-mediated apoptosis in p53-resistant oral SCC (HSC-4) cells[12]. Nevertheless, the mechanisms underlying p53 resistance have not been fully elucidated, and thus are still a subject of intensive studies.

Defect in Ser46 Phosphorylation and Resistance to p53-induced Apoptosis

To investigate resistance mechanisms against p53 gene transfer in oral SCC, we examined the responses of oral SCC cell lines lacking functional p53 to p53 gene transfer[12]. In this study, HSC-3 cells were found to be resistant to p53 gene transfer. Western blotting using phospho-specific p53 antibodies clearly revealed that the Ser46 phosphorylation of exogenous p53 is severely impaired in HSC-3 cells. In contrast, phosphorylations of both Ser15 and Ser46 were detected in the other cell lines examined, suggesting that the resistance to p53-mediated apoptosis depends on a defect in Ser46 phosphorylation. Consistent with this, the loss of Ser46 phosphorylation has also been observed in some tumor cells resistant to p53 gene transfer[77,78]. The resistance to p53-mediated apoptosis and tumor cell growth suppression in HSC-3 cells was overcome by the introduction of a mutant p53, termed p53S46D, that mimics Ser46 phosphorylation (Fig. 4). Conversely, a Ser46-phosphorylation defective mutant, p53S46A, failed to suppress tumor cell growth of p53-sensitive HSC-2 cells. These results provide evidence that the inability to phosphorylate Ser46 on p53 is responsible for the development of resistance to p53-mediated apoptosis in oral SCC.

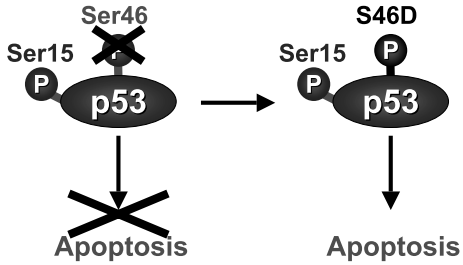

Fig. 4 Mutant p53S46D mimics phosphorylation to overcome the resistance to p53-mediated apoptosis in HSC-3 cells

Overexpression of wt-p53 is not sufficient to induce apoptosis in HSC-3 due to the defect of Ser46 phosphorylation. Introduction of the mutant (substituted Ser46 with Asp) termed p53S46D that mimics Ser46 phosphorylation restores p53-mediated apoptosis in HSC-3 cells.

While accumulating evidence has demonstrated the importance of Ser46 phosphorylation following severe DNA damage in p53-mediated apoptosis, recent studies have reported observations that p53 phosphorylation is dispensable for transcriptional activation and apoptosis, and that regulation of the intracellular level of p53 in the absence of Mdm2 is the major reason for N-terminal phosphorylation under physiological conditions[79—81]. Inhibition of the p53 Mdm2 interaction by either p14ARF, a negative regulator of Mdm2, or nutlin-3, an Mdm2 antagonist, is sufficient to stabilize endogenous wt-p53, and activate p53-dependent transcription and apoptosis in the absence of the major N-terminal phosphorylation, including Ser46. Furthermore, recent observations involving four different cell lines showed that Ser46 phosphorylation of p53 is not a prerequisite for adriamycin-induced apoptosis and the transactivation of pro-apoptotic genes[82]. Although the role of Ser46 phosphorylation in p53-mediated apoptosis is still controversial, however, our findings that phosphorylation mimicking the S46D mutant can overcome the defect of Ser46 phos-

phorylation and the p53-resistance of HSC-3 cells clearly indicate the critical role of this phosphorylation in the apoptotic function of p53 even under conditions where high levels of p53 are overexpressed by means of adenoviral p53. Consistent with this, it has been shown that the S46D mutant preferentially binds to the promoter region of the PTEN tumor suppressor gene encoding a negative regulator for cell survival, whereas the phosphorylation-defective S46A mutant targets the Mdm2 in preference to the PTEN promoter[40]. Further studies are required to elucidate the dysregulation of Ser46 phosphorylation in HSC-3 cells as well as in other Ser46-phosphorylation-deficient cells. We speculate that the upstream regulatory kinases, including HIPK2, PKCδ, and DYRK2, or cofactors, such as p53DINP1, might be abrogated in these cells.

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