이학석사학위논문

Transactivation domain phosphorylation of wild-type p53 inversely correlates with clinicopathological features of HCC

정상형 p53의 전사활성 도메인 인산화와 간암의 임상병리적 특성의 역비례 상관관계

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Abstract

Background & Aims: The p53 protein is a well-known tumor suppressor, and *p53* gene mutation is related to poor prognosis in most types of tumors, including hepatocellular carcinoma (HCC). Although the expression of wild-type (WT) p53 protein is increased in 47.8% (43/90) of HCC cases, the WT p53 expression does not correlate with the expression of p53 target genes, such as *p21* (*Cip1*). Thus, we investigated the functional status of p53 in WT p53-carrying HCCs.

Methods: We sequenced the *p53* gene in 115 HCC samples and classified each sample as WT p53 or mutant (Mut) p53. We analyzed p53 expression, p53 transactivation domain (TAD) phosphorylation, p21 expression, Bax expression and catalase expression in each HCC sample by immunoblot analysis. We investigated the obtained data using Spearman correlation analysis. The functionality of the TAD phosphorylation sites was confirmed by a p53 immunoblot analysis in HepG2, Huh-7, and Hep3B cells.

Results: The level of WT p53 protein was positively correlated with HCC grade but not with p21 protein expression (p=0.103) or portal vein invasion (p=0.288). Instead, p53 TAD phosphorylation in WT p53 HCCs was correlated with p21 protein expression (p<0.001) and inversely correlated with both portal vein invasion (p=0.001) and recurrence after surgical resection (p=0.004). The importance of TAD phosphorylation for p53 activity was confirmed by p53 target gene expression.

Conclusions:

The TAD phosphorylation was strongly associated with clinicopathological features and recurrence after surgical resection in WT p53 HCC. Thus, the TAD

phosphorylation may also be an independent prognostic indicator in HCC patients carrying WT p53.

Keywords: p53 mutation; p21; recurrence; hepatocarcinogenesis

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CONTENT

LIST OF ABBREVIASTIONS

HCC	hepatocellular carcinoma
WT	wild-type
Mut	mutant
TAD	transactivation domain
ATM	ataxia telangiectasia mutated
PFA	pifithrin- α
PI3K	phosphoinositide 3-kinase
ROS	reactive oxygen species

Introduction

Despite many scientific and clinical advances, the malignant tumor hepatocellular carcinoma (HCC) causes a high rate of mortality.[1] Generally, HCC is classified into four differentiation grades according to Edmondson's and Steiner's classification: well differentiated (grade I), moderately differentiated (grade II), poorly differentiated (grade III), and undifferentiated (grade IV).[2] HCC dedifferentiation correlates with increasingly aggressive behaviors including proliferation, invasion, distant metastasis, and treatment resistance.[3]

The p53 protein level is increased in more than 50% of HCCs, and it had been thought that p53 protein overexpression is caused mainly by *p53* gene mutations.[4, 5] However, only 10.3% - 32.8% of all HCC cases involve *p53* mutations, although the mutation rate is usually higher in other types of cancer.[6-9] Moreover, even in poorly differentiated grade III HCC, only 35% of the HCCs exhibit *p53* gene mutation.[9] Thus, it is now thought that *p53* gene mutation is not the major causative factor in HCC progression.[6, 9] Immunohistochemistry data have revealed that only 40% of p53-positive HCC cases exhibit *p53* gene mutations; these data also indicate that the p53 protein level is increased in the absence of mutation in many cases of HCC.[6, 10] However, the functional role of wild-type (WT) p53 up-regulation in HCC remains unclear.

The p53 protein is famous for its anti-tumorigenic effect because this

protein controls the cell cycle, cell apoptosis, or both.[11] Generally, p53 protein levels are very low in normal cells, but when the cell is confronted by stress, such as ultraviolet irradiation, reactive oxygen species (ROS), ionizing radiation or oncogenic activation, the p53 protein is stabilized by the addition of certain chemical groups, and its concentration in cells increases.[11, 12] Because of its essential role as a tumor suppressor, p53 has been called the "guardian of the genome."

Under stress conditions, the p53 protein level is increased, and p53 target gene expression is increased. In addition to the stabilization of the p53 protein, p53 phosphorylation at different sites also modulates p53 transcriptional activity.[12] Of the various phosphorylation sites, it has been found that phosphorylation in the transactivation domain (TAD) is most important for p53 transcriptional activity.[13-17] The relationship between TAD phosphorylation and p53 transcriptional activity *in vivo* has been probed by several p53 knock-in mouse lines with constitutive phosphorylation mimics at TAD sites.[12, 14, 15] Other modifications associated with increased p53 function also have been reported, such as acetylation and sumoylation.[12] Thus, p53 protein stabilization and p53 protein modifications, such as phosphorylation, are important for achieving the full potential of p53 function.

The most important function of p53 is the transactivation of target genes, although there is growing evidence for p53 functions beyond transcriptional activity. One of the most important p53 target genes is *p21*

(*Cip1*), which is critical for cell cycle arrest and the induction of apoptosis during stress conditions.[18, 19] p21 is a nuclear protein that induces cell cycle arrest in the G1 and G2 phases by inhibiting cyclin/cyclin-dependent kinase complexes and PCNA functions.[20, 21] Clinical studies showed that p21 serves as an independent prognostic factor for HCC patient survival, although p53 expression is not related to the survival of HCC patients.[6, 22]

In many cancer types, the overexpression of the p53 protein is mostly due to *p53* gene mutations that lead to p53 protein stabilization.[23, 24] Interestingly, however, the overexpression of p53 protein expression frequently observed without *p53* gene mutation in HCC. In this report, we show that the WT p53 expression does not correlate with p21 expression. Instead, p53 TAD phosphorylation is linked to p21 protein expression and clinicopathological features in WT p53 HCCs. The TAD phosphorylation level is inversely correlated with microvascular invasion and portal vein invasion, which are closely linked to HCC prognosis.[25] Moreover, the TAD phosphorylation level is also inversely correlated with recurrence after surgical resection. Thus, our data suggest that p53 TAD phosphorylation is a prognostic factor for WT p53 HCC.

Materials and Methods

Plasmids

Human *TP53* cDNAs were obtained from HepG2 cell lines and cloned into pCMV/Myc.[26] The pCMV/Myc-p53S15A, pCMV/Myc-p53S20A, and pCMV/Myc-p53S37A plasmids were generated by site-directed mutagenesis using the primers listed in Table S1.

Antibodies

Mouse monoclonal anti-p53 antibodies (Santa Cruz Biotechnology Inc), rabbit polyclonal anti-phospho-p53-ser15 antibody (Cell Signaling Technology Inc), rabbit polyclonal anti-phospho-p53-ser20 antibody (Cell Signaling Technology Inc), rabbit polyclonal anti-phospho-p53-ser37 antibody (Cell Signaling Technology Inc), rabbit polyclonal anti-p21 antibody (Cell Signaling Technology Inc), rabbit polyclonal anti-Bax antibody (Cell Signaling Technology Inc), rabbit polyclonal anti-catalase antibody (Abcam®), and mouse monoclonal anti-β-actin antibody (Sigma) were used.

Tissue specimens and histopathology

Tissue samples from 115 HCCs and corresponding non-HCC tissues were collected as surgical specimens from Severance Hospital, Yonsei University College of Medicine (Seoul, Korea) (from patients of age 52±11 years; range, 25–77; 96 male and 19 female; all HBsAg-positive). Informed consent was obtained

from each patient, and the Institutional Review Board of Yonsei University approved the tissue collection procedure. The tissues were snap-frozen in liquid nitrogen and stored at -70°C. All non-HCC liver tissues showed hepatitis B virus-associated chronic hepatitis or cirrhosis. Representative sections underwent routine histological evaluation for differentiation, tumor size, vascular invasion, portal vein invasion, and satellite nodule formation. Differentiation was graded according to Edmondson-Steiner criteria. Of the HCC samples, 28 were grade I, 28 were grade II, and 59 were grade III.[2]

Immunoblot analysis

Tissue and cell lysates were prepared, and immunoblotting was performed as previously described.[27] The band intensity was quantified using ImageMaster 2D Elite software 4.01 (GE Healthcare Life Sciences). For HCC tissue analyses, expression ratios were calculated by dividing the intensity of the tumor tissue signal by that of the matched non-tumor tissue. Ratios > 2 were scored as increased (↑), ratios between 0.5 and 2 were scored as not increased (↔), and ratios < 0.5 were scored as decreased (↓).

Cell culture

Unless otherwise noted, the reagents were obtained from Sigma. The human Hep3B (p53 null, hepatoma), Huh7 (Mut p53, hepatoma), and HepG2 (WT p53, hepatoblastoma) cell lines were cultured in Dulbecco's Modified

Eagle's Medium (DMEM) with 10% fetal bovine serum. In some experiments, the cells were incubated with various combinations of 300 μM H_2O_2, 10 μM KU-55933, 5 μM wortmannin and 10 μM pifithrin-α (PFA) for 4 hours. For siRNA experiments, the cells were transfected with p53 or control siRNA (ON-TARGETplus®, Dharmacon, Lafayette, CO) using Oligofectamine (Life Technologies Corporation). After a 48-hour transfection, protein expression was analyzed by immunoblotting.

Statistical analysis

Statistical analyses were performed using SPSS (Ver.12, SPSS Inc). The all relationship was estimated using Spearman correlation analysis. *P* values of less than 0.05 were considered statistically significant.

Results

Immunoblot analyses of p53, p21, and Bax in WT p53 HCC

It has been reported that p53 expression does not correlate with p21 expression in HCC even though the *p21* gene is a well-established target gene of p53.[28, 29] Among all of the examined HCCs, increased p53 protein expression was correlated with increased HCC grade, whereas p21 expression was not (Table 1). However, 10.3% - 32.8% of HCCs carry *p53* gene mutations,[6-9] which can perturb the correlation analysis of p53 expression and p21 expression because p53 mutants usually lose its function. Interestingly, in approximately 41.8% of all HCCs, increased p53 expression is not due to *p53* gene mutation-mediated p53 protein stabilization.[9] Therefore, we checked the correlation between p53 and p21 expressions in WT p53 HCCs to determine whether the correlation analysis was affected by the inclusion of HCCs with mutant *p53*. For this analysis, we divided the HCCs into WT p53 HCCs and mutant (Mut) p53 HCCs based on p53 gene sequencing analysis.[9] Our data showed that increased p53 protein expression was significantly correlated with increased HCC grade (p=0.005): increased p53 protein expression was observed in 28.6% of grade I tumors, 45.8% of grade II tumors, and 63.2% of grade III tumors (Table 2 and Fig. 1A). However, increased p21 expression was not significantly correlated with HCC grade (p=0.561): increased p21 expression was observed in 39.3% of grade I tumors, 29.2% of grade II tumors, and 31.6% of grade III tumors (Table 2

and Fig. 1A). As expected, the increased WT p53 protein expression in HCCs was not correlated with increased p21 expression (p=0.103) (Fig. 1B). In addition, the expression of Bax, another target gene of p53, was also not correlated with WT p53 expression (p=0.791) (Table 3). Our analyses indicate that p53 mutation is not reason for lack of correlation between p53 expression and target gene expression in HCC.

Table 1. Frequency of p53 and p21 expression patterns according to diff erentiation grade in HCC†

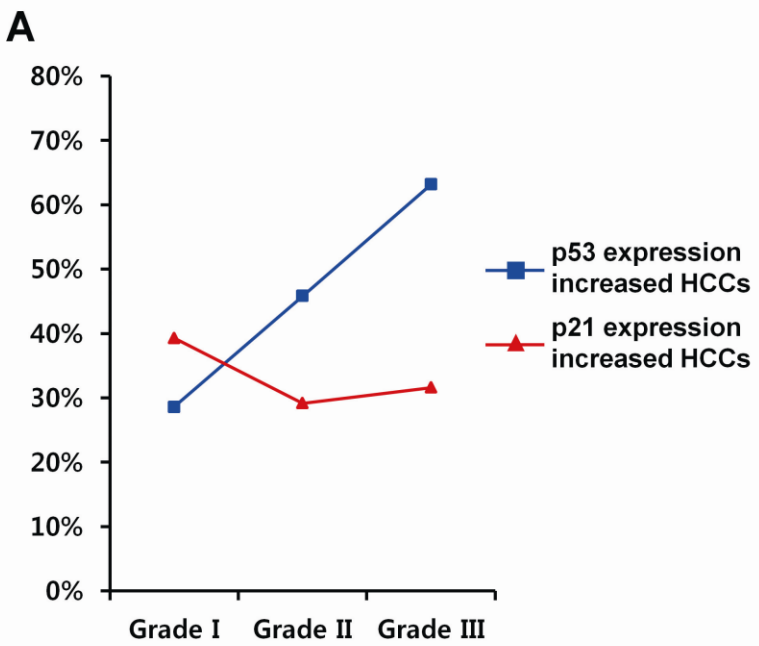
Protein expression		HCC Differentiation Grade			Total	*P*
		Grade I	Grade II	Grade III		
p53	↑	8	14	44	66	<0.001 *
	↔	20	14	15	49	
p21	↑	11	7	17	35	0.549
	↔	17	18	38	73	

†, protein expression intensity was interrogated by immunoblotting and quantitated by densitometry; n, number of cases; ↑, number of cases wit h a greater than 2-fold increase in expression in tumor tissue; ↔, numb er of cases with unchanged expression in tumor tissue; *, significant correlation (P<0.05); *P*, Spearman correlation.

Table 2. Frequency of p53 and p21 expression patterns according to diff erentiation grade in WT p53 HCC†

Protein expression		HCC Differentiation Grade			Total	*P*
		Grade I (n=28)	Grade II (n=24)	Grade III (n=38)	(n=90)	
p53	↑	8	11	24	43	0.005*
	↔	20	13	14	47	
p21	↑	11	7	12	30	0.561
	↔	17	17	26	60	

†, protein expression intensity was interrogated by immunoblotting and quantitated by densitometry; n, number of cases; ↑, number of cases wit h a greater than 2-fold increase in expression in tumor tissue; ↔, numb er of cases with unchanged expression in tumor tissue; *, significant correlation (P<0.05); *P*, Spearman correlation.



B

Protein Expression		p21 (Waf1/Cip1) ↑	p21 (Waf1/Cip1) ↔	Total	*P*
WT p53	↑	18	25	43	
	↔	12	35	47	
Sum		30	60	90	0.103

Figure 1. Expression **patterns of p53 and p21 in WT p53 HCC.** (*A*) The frequency of cases in which WT p53 and p21 were increased (↑) in each grade. (*B*) Correlation between p53 and p21 expression patterns in WT p53 HCCs.

Table 3. Correlation between p53 and Bax expression patterns in WT p53 HCC†

Protein Expression		Bax		Total	*P*
		↑	↔		
p53	↑	12	12	24	
	↔	14	12	26	
Sum		26	24	50	0.791

†, protein expression intensity was interrogated by immunoblotting and quantitated by densitometry; ↑, number of cases with a greater than 2-f old increase in expression in tumor tissue; ↔, number of cases with un changed or decreased expression in tumor tissue; *P*, Spearman correlation.

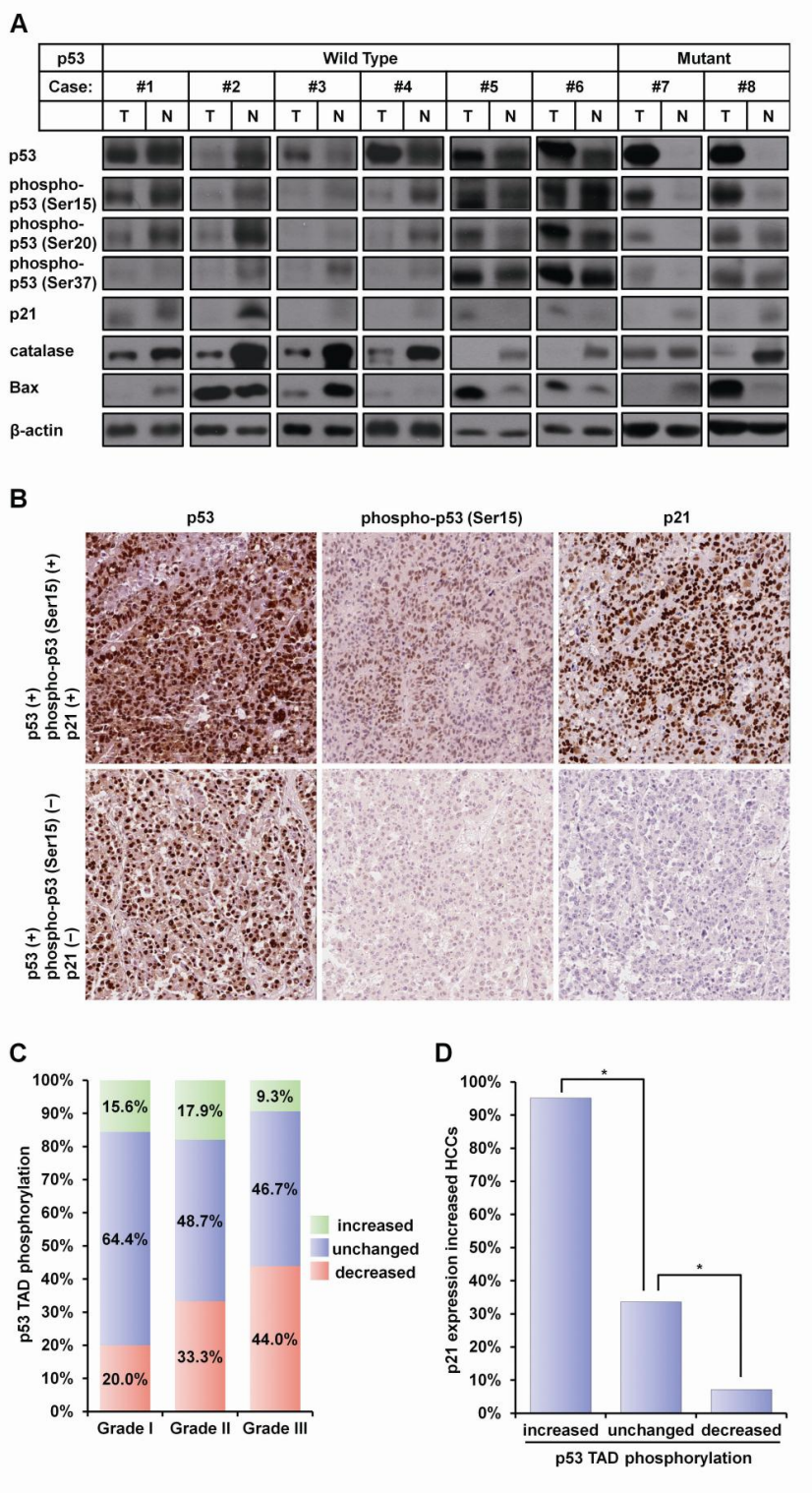
p53 TAD phosphorylation, but not overexpression, was correlated with p21 expression in WT p53 HCCs

The p53 protein has seven domains: an N-terminal TAD, a transactivation domain 2 (TAD2), a proline-rich domain, a DNA-binding domain, a nuclear localization signaling domain, a homo-oligomerization domain, and a C-terminal regulatory domain.[12] TAD phosphorylation is important for p53 functional activity, because it regulates the binding of p53 to cofactor proteins for the transactivation of target genes, as well as stabilizes the p53 protein by inhibiting its binding to MDM2.[12-15] Several other mechanisms are also suggested to stabilize the p53 protein, such as the acetylation of the p53 protein and decreased MDM2 sumoylation.[12]

In our data, although WT p53 expression levels were positively correlated with HCC grade, p21 expression was not correlated with HCC grade and WT p53 expression was not correlated with p21 expression. Thus, we hypothesized that WT p53 function might be reduced although WT p53 expression is increased in HCCs. In here, we analyzed the phosphorylation at three sites Ser (15, 20, 37) in the p53 TAD through immunoblot analyses with phospho-specific antibodies because TAD phosphorylation are most influential factor for p53 function.[13-17] The analyzed phosphorylation sites are known to be involved in p53 transcriptional activity. Representative expression patterns of p53, p53 TAD phosphorylation Ser (15, 20, 37), p21, catalase, and Bax in HCC

tissues are shown by immunoblotting (Fig. 2A) and representative expression patterns of p53, p53 TAD phosphorylation Ser 15, and p21 is shown by immunohistochemistry (Fig. 2B). These data revealed that the p53 protein expression increased concomitant with HCC grade, but the p53 phosphorylation at the tested residues did not increase with tumor grade (Table 4 and Fig. 2C). The p53 phosphorylation level was positively correlated with p53 expression in HCC grade I (p=0.001) (Table 5). However, phosphorylation was not correlated with p53 expression in HCC grade II or III tumors (p=0.433 and 0.620, respectively) (Table 5).

In addition, our analyses showed that the phosphorylation of WT p53 TAD Ser (15, 20, 37) was highly correlated with p21 expression (p<0.001) (Fig. 2D and Table 6) while WT p53 expression was not correlated with p21 expression (p=0.103) (Fig. 1B). However, the phosphorylation of Mut p53 TAD was not correlated with p21 expression (Table 7). Our findings suggest that WT p53-mediated target gene expression could be regulated by functional domain modifications, such as TAD phosphorylation, in HCC.


A
p53
Wild Type
Mutant
Case:
#1
#2
#3
#4
#5
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#8
T
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p53
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100%
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p21 expression increased HCCs
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Figure 2. Expression patterns of p53, phospho-p53 TAD, and p21 in HCC. (*A*) Representative examples of the expression of p53, phospho-p53 (ser15), phospho-p53 (ser20), phospho-p53 (ser37), p21, catalase, and Bax in HCCs. The immunoblots shown are from WT p53↔/p53 TAD↓/p21↔ (#1, #2), WT p53↑/p53 TAD↓/p21↔ (#3, #4), WT p53↑/p53 TAD↑/p21↑ (#5, #6), and Mut p53↑/p53 TAD↑/p21↔ (#7, #8) HCC tissues. (*B*) Immunohistochemistry of representative tissue samples, as defined in Table 3 (original magnification, ×400). (*C*) The frequency of cases in which p53 TAD phosphorylation was increased (↑), unchanged (↔), or decreased (↓) in each grade of WT p53 HCCs. (*D*) p21 expression with respect to p53 TAD phosphorylation status (increased (↑), unchanged (↔), or decreased (↓)) in WT p53 HCCs. *, significant correlation (P<0.05).

Table 4. Frequency of p53 TAD phosphorylation pattern according to differentiation grade in WT p53 HCC†

Phosphorylation		HCC Differentiation Grade			Total	*P*
		Grade I	Grade II	Grade III		
p53 TAD‡	↑	7	7	7	21	
	↔	29	19	35	83	
	↓	9	13	33	55	
Sum		45	39	75	159	0.009*

†, protein expression intensity was interrogated by immunoblotting and quantitated by densitometry; ‡, the number of p53 TAD phosphorylation cases includes the status of all 3 different phosphorylation sites (ser15, ser20 and ser37); ↑, number of cases with a greater than 2-fold increase in expression in tumor tissue; ↔, number of cases with unchanged expression in tumor tissue; ↓, number of cases with a greater than 2-fold decrease in expression in tumor tissue; *, significant correlation ($P<0.05$); *P*, Spearman correlation.

Table 5.

Correlation between p53 expression and p53 TAD phosphorylation patterns in grade I-III WT p53 HCC†

Protein expression		p53 TAD Phosphorylation‡			Total	*P*
		↑	↔	↓		
		Grade I HCC				
p53	↑	5	7	0	12	
	↔	2	22	9	33	
Sum		7	29	9	45	0.001*
		Grade II HCC				
p53	↑	5	8	6	19	
	↔	2	11	7	20	
Sum		7	19	13	39	0.433
		Grade III HCC				
p53	↑	3	23	19	45	
	↔	1	15	14	30	
Sum		4	38	33	75	0.620

†, protein expression or phosphorylation intensity were interrogated by immunoblotting and quantitated by densitometry; ‡, the number of p53 TAD

phosphorylation cases includes the status of all 3 different phosphorylation sites (ser15, ser20 and ser37); ↑, number of cases with a greater than 2-fold increase in expression in tumor tissue; ↔, number of cases with unchanged expression in tumor tissue; ↓, number of cases with a greater than 2-fold decrease in expression in tumor tissue; *, significant correlation (P<0.05); P, Spearman correlation.

Table 6. Correlation between p21 expression and p53 TAD phosphorylation patterns in WT p53 HCC†

Protein expression		p53 TAD phosphorylation‡			Total	*P*
		↑	↔	↓		
p21 (WAF1/Cip1)	↑	20	28	4	52	
	↔	1	55	51	107	
Sum		21	83	55	159	<0.001*

†, protein expression or phosphorylation intensity were interrogated by immunoblotting and quantitated by densitometry; ‡, the number of p53 TAD phosphorylation cases includes the status of all 3 different phosphorylation sites (ser15, ser20 and ser37); ↑, number of cases with a greater than 2-fold increase in expression in tumor tissue; ↔, number of cases with unchanged expression in tumor tissue; ↓, number of cases with a greater than 2-fold decrease in expression in tumor tissue; *, significant correlation (P<0.05); *P*, Spearman correlation.

Table 7. Correlation between p21 expression and p53 TAD phosphorylation patterns in Mut p53 HCC†

Protein expression		p53 TAD phosphorylation‡			Total	*P*
		↑	↔	↓		
p21 (WAF1/Cip1)	↑	4	1	1	6	
	↔	27	9	0	36	
Sum		31	10	1	42	0.532

†, protein expression or phosphorylation intensity were interrogated by immunoblotting and quantitated by densitometry; ‡, the number of p53 TAD phosphorylation cases includes the status of all 3 different phosphorylation sites (ser15, ser20 and ser37); ↑, number of cases with a greater than 2-fold increase in expression in tumor tissue; ↔, number of cases with unchanged expression in tumor tissue; ↓, number of cases with a greater than 2-fold decrease in expression in tumor tissue; *P*, Spearman correlation.

p53 TAD phosphorylation, but not overexpression, inversely correlated with clinicopathological features in WT p53 HCC

In addition to the correlation analyses of p53 TAD phosphorylation and p21 expression in HCC, we analyzed the correlation between WT p53 TAD phosphorylation and HCC clinicopathological features, such as microvascular invasion, portal vein invasion, and satellite nodule formation. In addition, we analyzed correlation between WT p53 TAD phosphorylation and recurrence after surgical resection. Previous reports had shown that p53 overexpression itself is not correlated with portal vein invasion in HCC.[5, 30] Our data show that increased WT p53 expression was positively correlated with only microvascular invasion (p=0.015) like *p53* gene mutation (p=0.002) (Table 8). However, interestingly, WT p53 TAD phosphorylation was inversely correlated with microvascular invasion (p=0.037) and portal vein invasion (p=0.001), while the TAD phosphorylation in WT p53 HCC was not correlated with satellite nodule formation (Table 8). Thus, in WT p53 HCC, p53 TAD phosphorylation status is more reliable indicator of clinicopathological features than p53 overexpression.

It has been known that portal vein invasion is one of the most important prognostic factors after surgical resection of HCC.[31, 32] In the above data, WT p53 TAD phosphorylation is inversely correlated with portal vein invasion. Thus, we analyzed correlation between WT p53 TAD phosphorylation and recurrence after surgical resection. The analysis showed that WT p53 TAD

phosphorylation was inversely correlated with recurrence after surgical resection (p=0.004) while the recurrence was correlated with neither p53 expression nor p53 mutation (Table 8). In addition, both increased p53 expression and p53 mutation were not correlated with portal vein invasion (Table 8). Therefore, our data clearly show that the TAD phosphorylation in WT p53 HCC is strongly associated with clinicopathological parameters and recurrence after surgical resection.

Table 8. Correlation between p53 status and tumor clinicopathological features in HCC†

		WT p53 expression		*P*	p53 gene mutation		*P*	p53 TAD phosphorylation‡			*P*
		↑	↔		Yes	No		↑	↔	↓	
Microvascular invasion	No	12	25	.015*	2	37	.002*	11	39	17	.037*
	Yes	31	22		23	53		10	44	38	
Portal vein invasion	No	35	42	NS	19	77	NS	21	76	42	.001*
	Yes	8	5		6	13		0	7	13	
Satellite nodules	No	30	40	NS	17	70	NS	20	55	41	NS
	Yes	13	7		8	20		1	28	14	
Recurrence after surgical resection§	No	11	4	NS	8	15	NS	6	12	6	.004*
	Yes	9	4		5	13		0	12	15	

†, protein expression intensity was interrogated by immunoblotting and quantitated by densitometry; ‡, the number of p53 TAD phosphorylation cases includes the status of all 3 different phosphorylation sites (ser15, ser20 and ser37); §, data includes only available cases among grade III HCCs; ↑, number of cases with a greater than 2-fold increase in expression in tumor tissue; ↔, number of cases with unchanged expression in tumor tissue; ↓, number of cases with a greater than 2-fold decrease in expression in tumor tissue; NS, not significant; *, significant correlation ($P<0.05$); *P*, Spearman correlation.

ROS-induced p53 TAD phosphorylation upregulates p21 expression in WT p53 HCC cells

Previous reports and our data show that ROS increases with increased HCC grade, and the expression of anti-oxidants, such as catalase, was decreased with increasing HCC grade (Table 9).[33] ROS usually induces genotoxicity, and increased levels of ROS activate p53.[34] In HCC tissue analyses, ROS levels and p53 protein levels both increase with increasing HCC grade and are correlated with each other. However, the expression of p53 target genes, such as p21, did not increase with increasing p53 expression, and our statistic analyses indicated that p53 TAD phosphorylation affected the p53-mediated expression of p21 in HCC.

We induced ROS in HCC-derived cell lines such as HepG2 (WT p53) and Huh-7 (Mut p53) by H_2O_2 treatment for 4 hours. The p53 TAD phosphorylation levels were increased by the H_2O_2 treatment without a significant increase in p53 protein level (Fig. 3A). In HepG2 cells (WT p53), p21 protein expression was increased concomitant with p53 TAD phosphorylation (Fig. 3A, left). However, in Huh-7 cells (Mut p53), p21 protein expression was not increased, although p53 TAD phosphorylation was increased (Fig. 3A, right). In addition kinase inhibitors that act upstream of p53, such as KU-55933 (ATM inhibitor)[35] and wortmannin (PI3K inhibitor)[36] inhibited ROS-induced p53 phosphorylation in both HepG2 and Huh-7 cells (Fig. 3B). Along with the defect

in p53 TAD phosphorylation, ROS-induced p21 protein expression was impaired (Fig. 3B). Moreover, ROS-induced p21 protein expression was also impaired by p53 DNA-binding inhibitors, such as PFA (Fig. 3C).

Our HCC tissue sample analyses data showed that all of three p53 TAD phosphorylation sites were correlated with *p21* gene expression. To investigate whether these phosphorylation sites are important for *p21* gene expression, Hep3B cells (p53 null) were complemented with either p53WT, p53S15A, p53S20A or p53S37A. After complementation, the cells were treated with H_2O_2 to generate ROS to induce p53 activation. H_2O_2 treatment did not increase p21 expression in non-complemented Hep3B cells (Fig. 3D, left). However, the treatment efficiently increased p21 expression after the complementation of Hep3B cells with the p53WT expression vector (Fig. 3D, right). In addition, our data showed that the TAD phosphorylation site mutants did not efficiently rescue p21 expression to the level observed in p53WT (Fig. 3E). These data indicate that p53 TAD phosphorylation is important for ROS-induced p53 target gene expression.

Table 9. Frequency of catalase expression pattern according to differentiat ion grade in WT p53 HCC†

Protein expression		HCC Differentiation Grade			Total	*P*
		Grade I	Grade II	Grade III		
Catalase	↔	13	4	6	23	
	↓	2	6	19	27	
Sum		15	10	25	50	<0.001 *

†, protein expression intensity was interrogated by immunoblotting and quantitated by densitometry; ↔, number of cases with unchanged expression in tumor tissue; ↓, number of cases with a greater than 2-fold decrease in expression in tumor tissue; *, significant correlation (P<0.05); *P*, Spearman correlation.

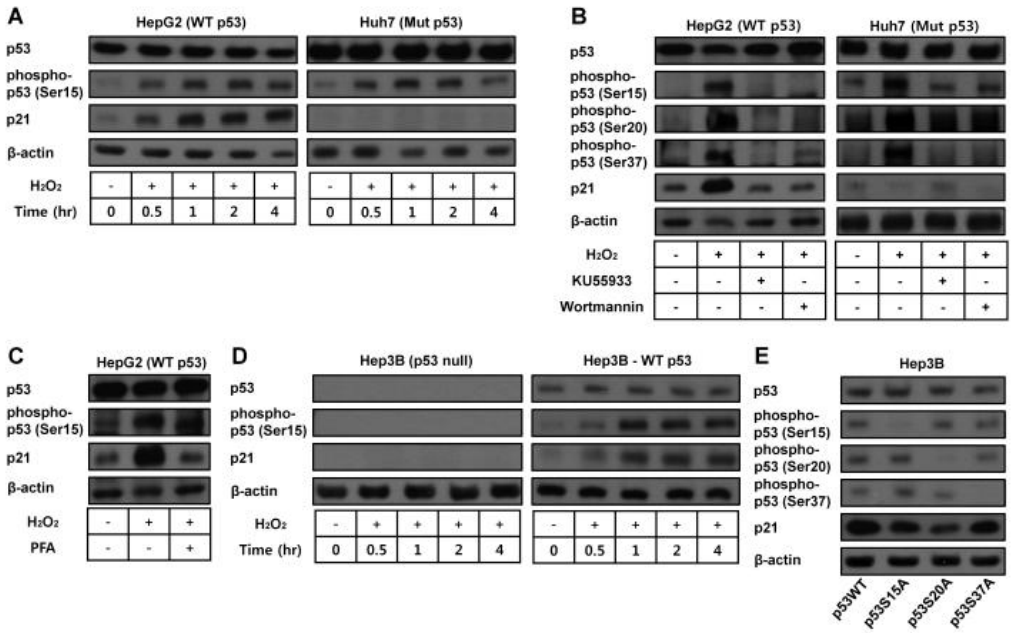

Figure 3. The ROS-induced phosphorylation of WT p53, but not Mut p53, increases the expression of p21 in HCC cells. (*A*) HepG2 and Huh-7 cells were treated with 300 μM H_2O_2 for 0.5, 1, 2, or 4 hours. Endogenous p53, phospho-p53 (ser15), and p21 protein levels were analyzed by immunoblotting. (*B*) HepG2 and Huh-7 cells were treated with 300 μM H_2O_2, 10 μM KU-55933, and/or 5 μM wortmannin for 4 hours. Endogenous p53, phospho-p53 (ser15), phospho-p53 (ser20), phospho-p53 (ser37), and p21 protein levels were analyzed by immunoblotting. (*C*) HepG2 cells were treated with 300 μM H_2O_2 and/or 10 μM PFA for 4 hours. Endogenous p53, phospho-p53 (ser15), and p21 protein levels were analyzed by immunoblotting. (*D*) Hep3B cells were treated with 300 μM H_2O_2 for 0.5, 1, 2, or 4 hours with or without transfection with pCMV/myc-p53. Endogenous p53, phospho-p53 (ser15), and p21 protein levels

were analyzed by immunoblotting. (*E*) Hep3B cells were transfected with pCMV/myc-p53WT, pCMV/myc-p53S15A, pCMV/myc-p53S20A, or pCMV/myc-p53S37A. After 48 hours, cells were treated with 300 μM H_2O_2 for 4 hours. p53, phospho-p53 (ser15), phospho-p53 (ser20), phospho-p53 (ser37), and p21 protein levels were analyzed by immunoblotting.

Discussion

It has been reported that the *p53* gene is mutated in 10.3% - 32.8% of HCCs. The frequent ("hot spot") mutations R249S and V157F, which result in functionally defective p53 proteins, were significantly associated with a worse prognosis for HCC.[37] An animal model showed that the reactivation of WT p53 produces efficient liver carcinoma regression.[38] Moreover, hepatocyte-specific *p53* gene-deficient mice develop spontaneous liver cancer.[39] Thus, previous reports indicate that the normal function of p53 is important for the prevention of HCC progression. However, in our study, the WT p53 expression level was positively correlated with HCC grade, although p53 is a well-known anti-tumor protein. In addition, p21 protein expression is not correlated with WT p53 expression, although *p21* gene is a well-known target of the p53 protein. The p53-p21 cell cycle pathway plays an important role in growth control, and the inappropriate deregulation of this pathway has been implicated in carcinogenesis.[40] Moreover, p21 has been suggested as an independent prognostic factor for HCC.[29]

p53 protein stability and activity can be regulated by protein modifications, such as phosphorylation and sumoylation. It has been suggested that these modifications also affect p53-mediated target gene expression. For example, p21 expression is impaired in tumors through p53 mutation or inactivation by destabilization.[41-43] However, in our analysis with HCC samples, the p53 mutation does not correlate with the p21 gene expression. In addition,

the increased level of WT p53 protein in HCC also does not correlate with the increased level of p21 protein. Instead, our data clearly showed that WT p53 TAD phosphorylation, rather than expression, was highly correlated with p21 expression. Moreover, our data showed that decreased level of WT p53 TAD phosphorylation was correlated with HCC grade. Therefore, there are other p53 inactivation mechanisms involved in HCC. Many in vitro studies have shown that p53 TAD phosphorylation events are important factors for p53-mediated target gene expression.[13, 44] In addition, several p53 TAD mutant mouse lines exhibited a decreased level of p53 transactivation activity.[12, 14, 15] Therefore, the p53 TAD phosphorylation level is an important factor of the p21 expression in HCC.

Previous reports have shown that p53 expression itself is not a reliable index for clinicopathological features in HCC.[4, 5] However, our data indicate that WT p53 TAD phosphorylation is inversely correlated with HCC portal vein invasion. It has been known that portal vein invasion is the most influential factor for prognosis of HCC and known as grave prognostic indicator in HCC.[31, 32] Thus, WT p53 TAD phosphorylation could be closely linked with prognosis of WT p53 HCC. Actually, our data showed significant inverse correlation between WT p53 TAD phosphorylation and recurrence after surgical resection. Therefore, WT p53 TAD phosphorylation is the useful prognostic factor for recurrence after surgical resection.

According to our data, WT p53 TAD phosphorylation might be

important for the inhibition of HCC progression. Actually, p53 TAD phosphorylation level is increased under the genotoxic conditions, such as exposure to ROS. Previous reports showed that ROS levels are positively correlated with HCC grade and that the levels of anti-oxidant enzymes, such as catalase, are negatively correlated with HCC grade.[33] Interestingly, in HCC, although the ROS level is increased, and the catalase expression level is decreased with increasing HCC grade, the phosphorylation of WT p53 TAD is not increased with increasing HCC grade. These data suggest that p53 activity is suppressed in HCC through a down-regulation of TAD phosphorylation, even under the genotoxic conditions induced by ROS. This may be accomplished through the inactivation of p53 upstream kinases, but the exact mechanism remains to be clarified.

In many tumors, the p53 mutation rate is high, and this accounts for p53 protein overexpression. However, in HCC, the *p53* gene mutation rate is only 35%, even in grade III HCC, and this frequency of mutation cannot explain the observed p53 overexpression. Prior to this report, *p53* gene mutation was the only factor considered in relationship analyses between p53 and HCC pathological features or prognoses. Thus, our data indicate that TAD phosphorylation level analysis is an important but previously overlooked component of WT p53 HCC evaluation.

In summary, our findings here have provided several novel insights into HCC pr ogression. When WT p53 TAD phosphorylation is not considered, there is no cor

relation between WT p53 expression and p21 expression in HCC. In addition, th e TAD phosphorylation is strongly associated with clinicopathological features a nd recurrence after surgical resection in WT p53 HCC, while the correlation bet ween p53 expression and clinicopathological features has been controversial. T hus, our data suggest that the TAD phosphorylation in WT p53 HCC is reliable pr ognostic factor for HCC progression. In addition, the elevation of WT p53 TAD p hosphorylation level could be considered as a useful therapeutic approach to HC C patients carrying low level of the TAD phosphorylation in order to provide imp roved clinical outcome.

Supplementary Tables

Table S1. Primers

Description	Sequence
pCMV/Myc-p53-S15A Forward	5'-ATCCTAGCGTCGAGCCCCCTCTG**GCT**CAGGAAACATTTTCAGACCTATGG-3'
pCMV/Myc-p53-S15A Reverse	5'-CCATAGGTCTGAAAATGTTTCCTG**AGC**CAGAGGGGGCTCGACGCTAGGAT-3'
pCMV/Myc-p53-S20A Forward	5'-CCCTCTGAGTCAGGAAACATTT**GCA**GACCTATGGAAACTACTTCCTG-3'
pCMV/Myc-p53-S20A Reverse	5'-CAGGAAGTAGTTTCCATAGGTC**TGC**AAATGTTTCCTGACTCAGAGGG-3'
pCMV/Myc-p53-S37A Forward	5'-CAACGTTCTGTCCCCCTTGCCG**GCC**CAAGCAATGGATGATTTGATGC-3'
pCMV/Myc-p53-S37A Reverse	5'-GCATCAAATCATCCATTGCTTG**GGC**CGGCAAGGGGGACAGAACGTTG-3'

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국문초록

연구 배경 및 목적: p53 단백질은 잘 알려진 종양억제유전자이며, p53 유전자의 돌연변이는 간암을 포함한 다양한 종류의 종양에서 나쁜 예후와 연관되어 있다. 그런데 47.8%의 간암 환자에서 정상형 p53의 발현이 증가되어 있음에도 불구하고, p21과 같은 p53에 의해 발현이 유도되는 유전자들의 발현은 증가되어 있지 않다. 이에 따라, 정상형 p53을 발현하는 간암에서 p53의 기능적 특성을 연구하였다.

연구 방법: 115명의 간암 환자에서 추출한 암조직에서 p53 유전자의 서열을 확인하여 정상형 p53 또는 돌연변이 p53을 발현하는 환자를 분류하였다. 그리고 p53의 발현, p53 전사활성도메인의 인산화, p21의 발현, Bax의 발현, 그리고 catalase의 발현 등을 면역블롯을 분석하였다. 그렇게 분석한 정보의 스피어만 상관계수를 구하였다. 또한 간암세포주인 HepG2, Huh-7, 그리고 Hep3B 세포주에서 면역블롯 분석을 통해 p53 전사활성도메인의 기능적 특성을 다시 한 번 확인하였다.

연구 결과: 정상형 p53의 발현 정도는 간암의 분화도와 양의 상관관계를 나타내었으나, p21의 발현 정도 및 간문맥 침윤 정도와는 상관관계를 나

타내지 않았다. 대신 p53 전사활성도메인의 인산화가 p21 발현과 양의 상관관계를 가지며, 간문맥 침윤 및 간암절제 후 재발과 음의 상관관계를 나타냈다. 또한 p53의 활성을 위해 전사활성도메인의 인산화가 중요함을 p53 타깃 유전자 발현 측정을 통해 다시 한 번 확인하였다.

연구 결론: 정상형 p53을 발현하는 간암에서 p53 전사활성도메인의 인산화는 간암의 임상병리학적 특성 및 절제 후 재발 현상과 강하게 연관되어 있다. 그러므로, p53 전사활성도메인의 인산화가 정상형 p53을 가진 간암환자에 대한 독립적인 예후 판단 지표로서 사용될 수 있을 것으로 생각된다.

주요어: p53 돌연변이, p21, 암 재발, 간암 발달 과정

학번: 2010-20312

감사의 글

생각보다 많이 힘들었지만, 값진 시간이었던 석사생활을 마무리하며, 소중한 분들께 이렇게 감사의 글을 쓰게 되어 정말 기쁩니다.

먼저 대학원 생활을 잘 할 수 있도록 지도해주신 정구홍 교수님께 정말 감사 드립니다. 쉽지 않은 시간이었지만, 교수님께서 끊임없는 열의로 지도해주신 덕분에 석사 과정을 잘 마칠 수 있었습니다.

또한 제가 걱정 없이 공부만 할 수 있도록 지원해 주신 가족들에게 고마움을 전하고 싶습니다. 집에도 자주 들어가지 않고 효도 한 번 제대로 하지 못하였지만 항상 제가 공부하는 걸 응원해주시고 제 몸 걱정 하시며 보양식 한 번이라도 더 먹이려고 해주셨던 아버지, 어머니, 그리고 누나에게 정말 고맙고 사랑한다고 말하고 싶습니다.

공부하는 동안 가족 이상으로 긴 시간을 함께 했던 연구실 식구들, 특히 2년 반 동안 좋은 일도 나쁜 일 모두 함께하며 적절한 질타와 도움을 주었던 소연이에게 가장 먼저 고맙단 말을 전하고 싶습니다. 앞으로 우리 둘 다 박사과정은 서로 도우며 더 잘 해낼 수 있기를 바랍니다. 현재 실험실의 방장을 맡아 MGL의 대들보 역할을 해주고 있는 승원이형, 지금까지도 그래왔고 앞으로도 계속 같이 좋은 실험실이 되도록 도와주시길 부탁 드리며 매우 감사 드립니다. 새로 입학한 희원이와 윤식이형 둘 다 좋은 연구를 하여 훌륭한 논문을 쓰게 되길 바랍니다. 학부생이지만 오랫동안 연구실에서 함께하며 다양한 주제로 토론 및 생각을 할 수 있게 해준 민형이에게도 매우 고맙습니다. 그리고 오랜 연구 경험을 통해 제가 생각지 못했던 조언을 해주시는 은경누나에게도 정말 고맙습니다. 마지막으로 현재 광주과기원 교수로 부임하셔서 멀리서도 좋은 지도를 해주시는 박성규 교수님과 지금은 졸업하고 연구실을 나갔지만 짧은 동안 집약적으로 좋은 연구 지도 및 조언을 해주신 임승외 박사님과 민지영, 김혜림, 심희연 선배님들에게도 고맙단 말을 전하고 싶습니다.

스스로는 잘 몰랐겠지만 제가 공부하면서 힘들 때마다 항상 위로가 되어주고 언제나 듣기 좋은 목소리로 응원해주었던 이지은 씨, 그리고 매일 같이 다양한 방법으로 저를 응원해 주고 힘이 되어준 친구들 김경진, 송한수,

김대준, 김선욱, 김영헌, 김재홍, 김성년, 박현서, 김연수, 김종규, 서동권, 영진형, 정환형, 한솔이, 한량형 모두에게 고맙고 다들 하는 일이 모두 잘 되길 바랍니다.

감사의 글을 쓰면서 석사과정 동안 있었던 일들이 주마등처럼 흘러가는 것은 누구나 비슷할 것 같습니다. 앞으로 이 순간을 떠올리며, 계속해서 박사과정을 더 열심히 해나갈 수 있는 원동력이 되도록 하겠습니다. 항상 좋은 일이 가득하시길 바랍니다. 감사합니다.

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