Int J Clin Exp Pathol 2015;8(11):14995-15000
www.ijcep.com /ISSN:1936-2625/IJCEP0016371

Original Article

Association of RNF43 with cell cycle proteins involved in p53 pathway

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Received September 18, 2015; Accepted October 24, 2015; Epub November 1, 2015; Published November 15, 2015

Abstract: Our previous study has demonstrated that RNF43 could regulate the cell cycle in a p53-dependent manner in HCC. In this study, we aimed to access whether RNF43 could interact with cell cycle proteins involved in p53 pathway, including pRB, Cyclin D1 and MDM2. Totally, 123 paired HCC tissues and corresponding noncancerous tissues from HCC patients were included, and the expression of Cyclin D1, pRB and MDM2 was analyzed using tissue microarray. Our results showed the expression level of RNF43 in HCC was positively correlated with that of MDM2, Cyclin D1 and pRB-S780. There was no significant correlation between the expression of RNF43 and pRB-S807/S811. Indicating that RNF43 effected cell cycling by regulating the expression of pRB, Cyclin D1 and MDM2 proteins, and pRB-S780 but not pRB-S807/S811, was participated in RNF43 regulated cell cycling.

Keywords: HCC, RNF43, p53, cell cycling, tissue microarray

Introduction

Hepatocellular carcinoma (HCC) is among the most frequent malignancies worldwide [1]. Though therapies have been improved in these years, the survival of HCC patients is still poor [2]. It is important to understand the biological mechanisms of HCC and finally improve the treatment for HCC patients.

P53 is a commonly studied tumor suppressor protein. The most important role of p53 is the response to multiple stresses (such as chromosome breakage, oxygen deficiency and cancerous signaling) [3]. P53 exerts its function mainly by specifically binding to the conserved region 5'-RRRCWWGYYY-(separated by 0 to 13 base pairs)-RRRCWWGYYY-3' (R represents A or G, W represents A or T, Y represents C or T, N could be any base), and regulating the transcriptional activities of other genes [4]. As an important tumor suppressor, p53 regulates various of cancerous related genes which play important roles in cellular apoptotic, proliferation and invasive processes [5].

Plenty of researches have demonstrated the tumor suppressor role of p53 in the progression of carcinogenesis. Among the human genome, p53 is recognized to be the most likely mutated gene in cancers: nearly 50% of the cancers bear mutant p53 [6]. It has been reported that the mutation of p53 could promote the cancerous processes such as proproliferation, apoptotic suppression, and cellular invasion [7]. The association of p53 and RNF43 has been demonstrated recently. It has been reported that RNF43 could bind to NEDD-4-like ubiquitin-protein ligase-1 (NEDL1), and enhanced the pro-apoptotic activity of cells in a p53 dependent manner [8]. Furthermore, RNF43 could also interact with nucleoprotein (NP), by which it facilitated the ubiquitination of p53 and inhibited NP-driven activation of p53 transcription [9].

In our previous study, we have demonstrated that RNF43 acted as an oncogene in HCC and involved in the processes of cellular proliferation, anti-apoptosis, invasion and tumorigenesis. Knockdown of RNF43 could enhance the

Table 1. General Characteristics of the HCC patients

Clinicopathologic variable	Count
Gender	
Male	110
Female	13
Age (years)	
≤50	73
>50	50
Liver cirrhosis	
Absence	54
Presence	69
Maximal tumor size (cm)	
≤5	33
>5	90
Capsular formation	
Absence	119
Presence	4
Tumor nodule number	
Solitary	88
Multiple (≥2)	35
Vascular invasion	
Absence	92
Presence	31
Serum AFP (ng/mL)	
≤400	67
>400	56
HBsAg	
Negative	11
Positive	112
Anti-HCV	
Negative	123
Positive	0

expression of p53 and inhibited the malignant phenotype of HCC cells. Protein-protein interaction network demonstrated that p53 was the center hub of RNF43 regulated network, indicating RNF43 exerted its function through a p53 dependent pathway [10]. In this study, we analyzed the expression of p53 pathway proteins involved in cell cycling (including MDM2, Cyclin D1 and pRB), and tried to find whether RNF43 could regulate the cell cycle with these p53 associated proteins.

Material and methods

Patients

Paired HCC tissues and corresponding noncancerous tissues from 123 HCC patients who received hepatic resection were enrolled. All of these patients received operation in our hospital between 2007 and 2011. The diagnoses of HCC were confirmed by pathology. All the patients have provided written informed consent, and the local ethics committee approved this study. The general characteristics of the patients are shown in **Table 1**.

Immunohistochemistry

The expression of Cyclin D1, pRB and MDM2 were detected using immunohistochemistry tissue microarray. Briefly, tissue samples were fixed in 10% formalin for 1 to 7 days. Citric acid buffer in microwave oven was used for antigen retrieval. The primary antibodies used are listed in **Table 2**. The tissue microarray was then incubated with HRP-conjugated secondary antibody (Invitrogen) at 37°C for 1 hour. Finally, the immunoreactivity was visualized with DAB (Zhongshan Goldbridge Biotechnology). Slides were analyzed on Olympus IX70 microscope. Cases that showed detachment from the tissue microarray were excluded.

Statistical methods

Statistical analysis was performed using SPSS 16.0 software (SPSS, Chicago, IL, USA). To evaluate the expression association of RNF43 and p53 correlated proteins, Pearson's bivariate correlation test was applied. Paired Student's t test was used to compare mean values between two groups. Statistical significance was accepted if $P<0.05$.

Results

Expression levels of RNF43 and p53 related cell cycle proteins in HCC

To analyze the expression levels of RNF43 and p53 related proteins in HCC tissues, 123 patients who underwent hepatic resection for HCC were enrolled for tissue microarray analysis. Expression intensity was graded according to the percentage of the stained cells. For the expression of RNF43: 0 (negative), 1+ (≥0% to <25%), 2+ (≥25% to <75%), 3+ (≥75%). As the expression levels of MDM2, Cyclin D1 and pRB were much lower than that of RNF43, the grades of the expression levels of these proteins were : 0 (negative), 1+ (≥0% to <5%), 2+ (≥5% to <25%), 3+ (≥25%). The results showed that RNF43 was over-expressed in HCC tissues

Table 2. Primary antibodies used in this study

Proteins	Antibodies	Dilution	Incubate
RNF43	#HPA008079, Sigma-Aldrich	1:200	Over-night
MDM2	#S1357, Epitomics	1:100	Over-night
Cyclin D1	#2978, cell signaling	1:100	Over-night
pRB (S780)	#8095-1, Epitomics	1:100	Over-night
pRB (S807/S811)	#9308s, cell signaling	1:100	Over-night

Table 3. Expression Level of RNF43 and p53 related proteins in HCC and corresponding normal tissues

Clinicopathologic variable	Expression Levels[#]				
	0	1+	2+	3+	*P* value
RNF43					<0.001*
Cancer	5	7	41	42	
Normal	22	19	40	14	
MDM2					<0.001*
Cancer	1	3	3	88	
Normal	23	31	21	20	
Cyclin D1					0.342
Cancer	44	29	7	17	
Normal	55	20	5	17	
pRB-S780					<0.001*
Cancer	15	15	12	51	
Normal	57	18	10	8	
pRB-S807/S811					<0.001*
Cancer	46	18	18	12	
Normal	80	8	5	1	

[#]Expression was graded according to the percentage of the stained cells. For the expression of RNF43: 0 (negative), 1+ (≥0% to <25%), 2+ (≥25% to <75%), 3+ (≥75%). For the expression of MDM2, Cyclin D1 and pRB: 0 (negative), 1+ (≥0% to <5%), 2+ (≥5% to <25%), 3+ (≥25%). *P < 0.05. Cases that showed detachment from the tissue microarray were excluded.

compared with that of the corresponding normal tissue ($P = 1.12*10^{-9}$). We also showed the expression levels of MDM2 ($P = 2.39*10^{-24}$) and pRB ($3.98*10^{-18}$ for pRB-S780 and $4.06*10^{-10}$ for pRB-S807/S811) were up-regulated in HCC tissues (**Table 3**). Representative figures were presented in **Figure 1**.

Association of the expression levels of RNF43 and p53 related proteins

Pearson's bivariate correlation test was applied to analyze the association between RNF43 and p53 related proteins (**Table 4**). Our results showed that the expression of RNF43 was positively correlated with the expression of MDM2 (P = 0.003) Cyclin D1 (P = 0.022) and pRB-S780 (P = 0.008). There was no significant correlation between the expression of RNF43 and that of pRB-S807/S811 (P = 0.437).

Discussion

As a tumor suppressor, p53 can suppress the cellular proliferation and anti-apoptotic processes, and facilitates the response to cellular and environment stresses to protect the genomic integrity [11]. Our previous study demonstrated that RNF43 could regulate the cell cycle in a p53-dependent manner [10]. To further clarify the involvement of p53 related proteins in RNF43 regulated cell cycling, we analyzed the expression association of RNF43 and p53 related proteins MDM2, Cyclin D1 and pRB.

Our results showed that the expression of RNF43 was positively correlated with the expression of MDM2 (P = 0.003). It has been demonstrated that RNF43 could interact with nucleoprotein (NP) and decreased the stabilization of p53 though the MDM2 dependent pathway [9]. The binding of MDM2 to p53's N-terminus could suppress the transcriptional regulatory activity of p53, and could also facilitate its poly-ubiquitination and proteasome-mediated degradation [12]. Reversely, p53 could also enhance the transcriptional activity of MDM2 by binding to the promoter sequence of MDM2 [13], and form an RNF43-p53-MDM2 negative feedback loop. But whether RNF43 could regulate the expression level or actively of MDM2 directly has not been reported yet.

Cyclins are structurally conserved proteins that bind to and induce the activity of Cyclin dependent kinases (Cdks) [14]. The expression levels of Cyclins are mainly mediated in an APC/C dependent manner [15]. Cyclin D is a number of Cyclins that could form complexes with four Cdks: CDK2, CKD4, CDK5, and CDK6 [16], which plays an important role in cell proliferation. In this study, we found that the expression of RNF43 was positively correlated with that of Cyclin D1. The most likely mechanism is that RNF43-incduced inhibition of p53 suppressed the expression of p21 and then induced the activity of Cyclins/CDKs complexes [17]. It has

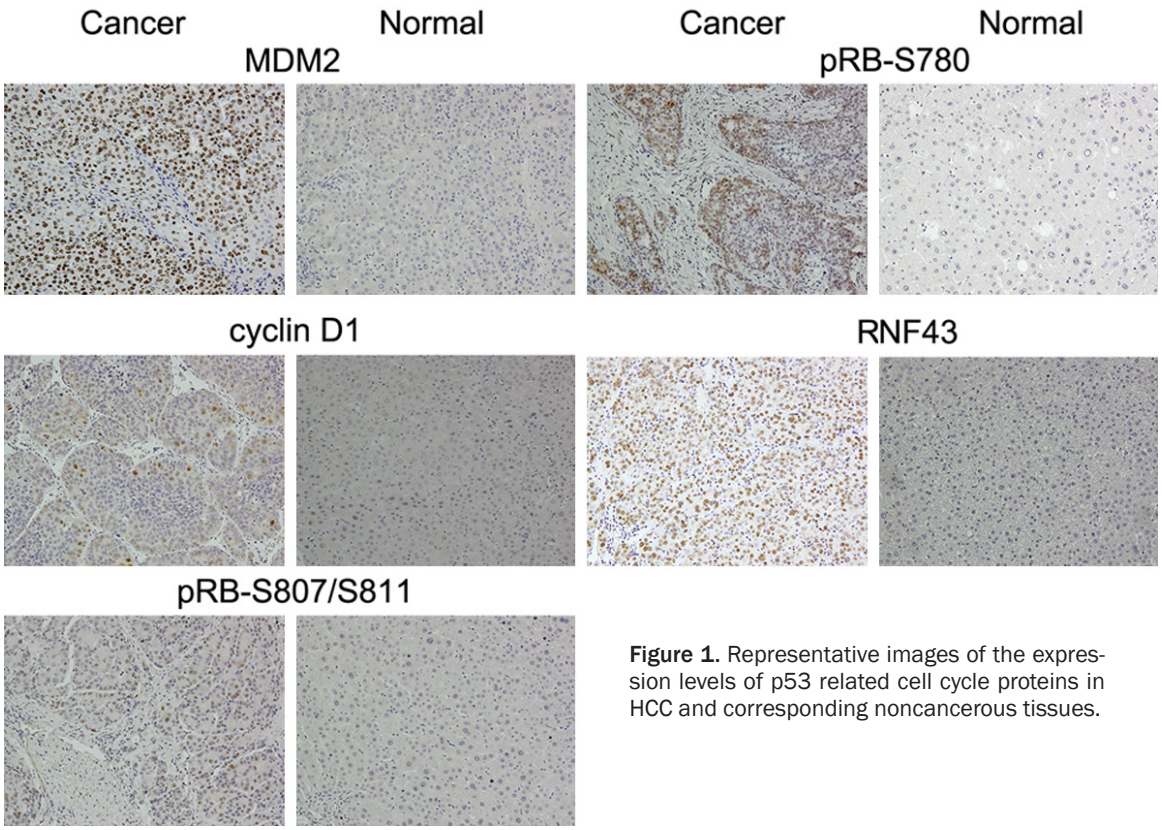

Figure 1. Representative images of the expression levels of p53 related cell cycle proteins in HCC and corresponding noncancerous tissues.

Table 4. Correlations between the expression of RNF43 and p53 related proteins

Clinicopathologic variable	R value	*P* value
RNF43	0.296	0.003*
MDM2		
RNF43	0.229	0.022*
Cyclin D1		
RNF43	0.263	0.008*
pRB-S780		
RNF43	0.079	0.437
pRB-S807/S811		

#Expression was graded according to the percentage of the stained cells. For the expression of RNF43: 0 (negative), 1+ (≥0% to <25%), 2+ (≥25% to <75%), 3+ (≥75%). For the expression of MDM2, Cyclin D1 and pRB: 0 (negative), 1+ (≥0% to <5%), 2+ (≥5% to <25%), 3+ (≥25%). *P < 0.05. Cases that showed detachment from the tissue microarray were excluded.

also been demonstrated that p53 could down-regulate the protein level of Bcl-3. Inhibited Bcl-3 suppressed the expression levels of p52 and NF-κB, and inhibited the binding of p52/NF-κ complexes to the promoter region of the Cyclin D1, and then attenuated the transcriptional activity of Cyclin D1 [18]. Furthermore, suppressed p53 could attenuate the transcription of miR-365, which could bind to the 3' UTRs of Cyclin D1, thus inducing the expression of Cyclin D1 [19, 20].

Finally, we found that the expression level of pRB was commonly up-regulated, and this up-regulation was positive associated with the overexpression of RNF43. The retinoblastoma (RB) has been widely recognized as a tumor suppressor gene, which is frequently phosphorylated to lose its activity in many cancers. The most important role of Rb is to arrest the progress of cell cycle at G1 phase. When a cell is ready for G1/S transition, CDK/Cyclins complexes phosphorylate Rb to its inactive form pRb [21]. S780 and S807/S811 are of the most common phosphorylation sites of the RB protein. It has been demonstrated that Cyclin D/Cdk4 could specifically phosphorylate the serine at residue 780 (S780) of RB protein in G1 phase in a cell cycle-dependent manner [22]. Cyclin D/Cdk4 could also phosphorylate S807/S811. It has been reported that phosphorylated S807/S811 could facilitate the phosphorylation of other sites of the RB protein [23]. In this study, we have found that the phosphorylation

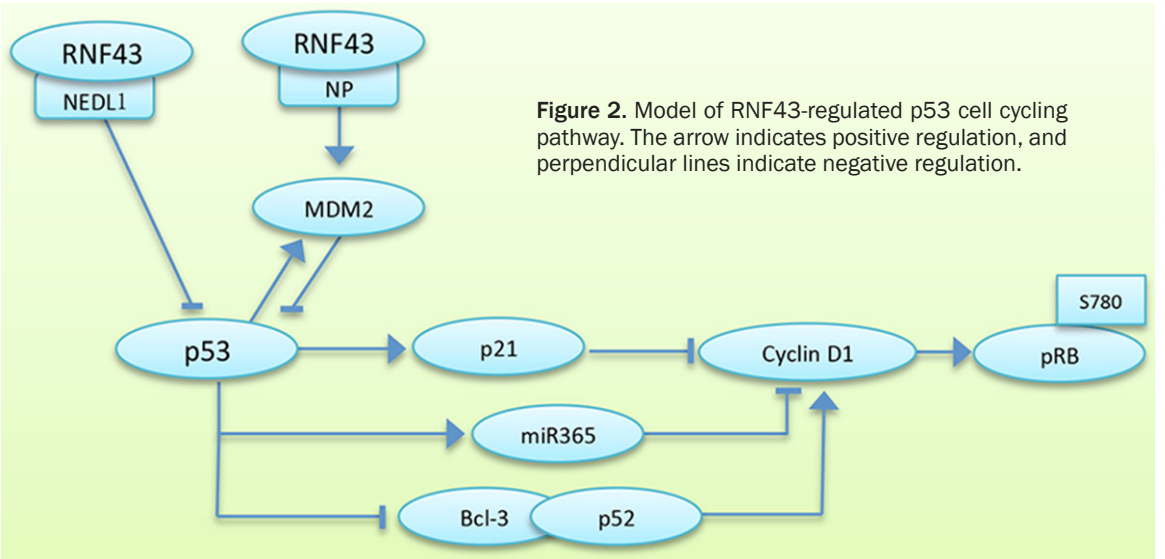

Figure 2. Model of RNF43-regulated p53 cell cycling pathway. The arrow indicates positive regulation, and perpendicular lines indicate negative regulation.

of S780 but not S807/S811 was associated with RNF43 expression.

In conclusion, our study exhibited the expression patterns and correlation of RNF43 and p53 pathway proteins involved in cell cycling. The most probable mechanism of RNF43-regulated p53 cell cycling pathway was shown in **Figure 2**. Briefly, RNF43 inhibited p53 in a MDM2 dependent or independent manner, and then down-regulated the expression of p21. Down-regulated p21 induced the activity of Cyclin D1 and the phosphorylation of pRB-S780. These data provide valuable information for better understanding of the association of RNF43 and p53 in HCC.

Acknowledgements

This study was supported by grants from the Zhejiang Provincial Natural Science Foundation (LY13H160004), grants from the National Natural Science Foundation of China (81502012), Chinese High Tech Research & Development (863) Program (2012AA020204), and National S&T Major Project (No. 2012ZX10002017).

Disclosure of conflict of interest

None.

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