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Overexpression of MDM2 protein in intrahepatic cholangiocarcinoma: relationship with p53 overexpression, Ki-67 labeling, and clinicopathological features

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Abstract Aberration of the *p53* gene is thought to be the most frequent genetic alteration in human cancers. Tp53 protein may be inactivated by the binding of the MDM2 protein. MDM2, the product of the *mdm2* gene, is an oncoprotein that binds to Tp53 and inhibits the p53-mediated transactivation. MDM2 overexpression has been reported in several human cancers, but not in intrahepatic cholangiocarcinoma (ICC). Therefore, we have evaluated the immunohistochemical overexpression of MDM2 and the relationship between its expression and histological grade, clinicopathological features, Tp53 overexpression, and Ki-67 labeling index in 47 cases of ICC. MDM2 and Tp53 were found to be overexpressed in 38% and 57% of the tumor, respectively. MDM2 and Tp53 were not expressed in non-tumorous liver tissue. There was no significant difference between the MDM2 overexpression and ICC tumor grade. However, MDM2 overexpression correlated with the presence of metastases ($P<0.01$) and advanced tumor stage ($P<0.05$). MDM2 overexpression also correlated with Tp53 overexpression ($P<0.03$) and Ki-67 labeling index ($P<0.03$). Our findings suggest that MDM2 overexpression may play a role in the late stage of human ICC.

Key words Intrahepatic cholangiocarcinoma · MDM2 Tp53 · Ki-67 · Clinicopathological correlation

Introduction

MDM2, the product of the *mdm2* gene, is an oncoprotein that binds to Tp53 and inhibits the p53-mediated transactivation [1, 5, 7, 20, 21, 29, 31, 34, 37, 38, 39]. The *mdm2* gene is mapped to chromosome 12q13–14 [10]. The transcription of the *mdm2* gene is activated by Tp53, the product of the *p53* gene. MDM2 directly binds to Tp53 and forms a negative-feedback loop that limits the growth-suppressing activity of Tp53 [53]. In response to DNA damage, wild-type Tp53 rapidly increases and induces *mdm2* gene expression and an accumulation of MDM2 [32, 37]. The induced MDM2 protein results in a reduction in Tp53 level through promoted proteosome-dependent degradation [13, 21, 22]. Moreover, MDM2 inhibits p53-mediated apoptosis and masks the transactivation domain of Tp53, so that Tp53 cannot interact with the transcriptional machinery, thus preventing p53-dependent apoptosis and transcription [4, 12, 18]. In addition, it has been reported that MDM2 inhibits the G1-phase blocking effect of retinoblastoma (Rb) protein [28, 56].

The relationship between aberration of Tp53 and MDM2 expression has been reported in several human cancers [2, 9, 10, 24, 25, 27, 41, 42, 43, 45, 46, 52]. For example, MDM2 overexpression was detected in 50% of patients with human breast cancer and showed a significant correlation with Tp53 overexpression and shortened patient survival [11]. In human liver malignancies, it has been reported that *mdm2* gene amplification has not been found in hepatoblastomas in which *p53* gene mutations were present [36]. Moreover, in human hepatocellular carcinoma, it has been suggested that expression of MDM2 may be related to tumor invasiveness through inactivating the tumor-suppressor function of the *p53* gene [41]. In human intrahepatic cholangiocarcinoma (ICC), although a few studies are available on the relationship between tumor progression and overexpression of Tp53 or Ki-67 labeling [35, 44, 48], there have been, to the best of our knowledge, no reports on MDM2. In the present study, we have therefore examined the overexpression of MDM2 in ICC and its relationship to Tp53 overexpression, Ki-67 labeling, and clinicopathological features.

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Materials and methods

Tissues

Specimens were retrieved from 47 cases of ICC from the surgical (21 cases) and autopsy (26 cases) archives in our laboratory. Informed consent was obtained from every patient. The age of the subjects ranged from 19 years to 81 years, with an average of 64 years. The male to female ratio was 27:20. The non-neoplastic liver tissue was normal in all cases. In each case, three to ten tissue specimens from the tumor and two to five tissue specimens from the non-tumorous liver were obtained. Among these specimens of each ICC case, we selected one representative specimen of ICC that was devoid of necrosis or artificial changes and one specimen of non-tumorous liver near the ICC; these two specimens of each ICC case were subjected to immunohistochemical examination. Specimens from extrahepatic metastases were also obtained, when available. All tissue specimens were fixed in 10% neutral buffered formalin and then embedded in paraffin. Three-micron serial sections were cut from each of the paraffin blocks. One section was stained with hematoxylin and eosin for tumor grading, and another with elastica van Gieson for assessment of ICC vascular invasion. The remaining sections were subjected to immunohistochemical staining. In two cases, fresh specimens from the tumor and non-neoplastic tissue of the same case were snap-frozen in liquid nitrogen and stored at –80°C until used for Western blotting.

Evaluation of metastatic spread was performed using X-ray, ultrasound sonography, computed tomography, and magnetic resonance imaging in surgical cases and using meticulous macroscopic and microscopic examination in autopsy cases. In total, 22 of 47 ICC cases showed metastases, the sites of which were lung (15 cases), lymph node (14 cases), adrenal gland (4 cases), gastrointestinal organs (4 cases), kidney (2 cases), brain (2 cases), bone (2 cases), and peritoneum (1 case). Some clinical and histopathological data of the ICC patients are shown in Table 1 and Table 2.

Table 1 Relationship between overexpression of MDM2 and clinicopathological features in 47 cases of intrahepatic cholangiocarcinoma. *well* well differentiated; *mod* moderately differentiated; *por* poorly differentiated; *NS* not significant. Statistics: χ^2 test

Categories	MDM2 positive cases		*P* value
Histology	Well	6/18 (33%)	
	Mod	7/19 (37%)	NS
	Por	5/10 (50%)	
Metastasis	Absent	5/29 (17%)	<0.01
	Present	13/18 (72%)	
Vascular invasion	Absent	6/12 (50%)	NS
	Present	12/35 (34%)	
Tumor stage	I	2/4 (50%)	
	II	1/11 (9%)	
	III	1/6 (17%)	<0.05
	IVA	1/4 (25%)	
	IVB	13/22 (59%)	

Table 2 Relationship between overexpression of Tp53 and clinicopathological features in 47 cases of intrahepatic cholangiocarcinoma. *well* well differentiated; *mod* moderately differentiated; *por* poorly differentiated; *NS* not significant. Statistics: χ^2 test

Categories	Tp53 positive cases		*P* value
Histology	Well	9/18 (50%)	
	Mod	11/19 (57%)	NS
	Por	7/10 (70%)	
Metastasis	Absent	14/25 (56%)	NS
	Present	13/22 (59%)	
Vascular invasion	Absent	7/12 (58%)	NS
	Present	20/35 (57%)	
Tumor stage	I	3/4 (75%)	
	II	6/11 (55%)	
	III	3/6 (50%)	NS
	IVA	2/4 (50%)	
	IVB	13/22 (59%)	

Immunohistochemistry

Consecutive sections of all tissue specimens were immunohistochemically stained for MDM2, Tp53, and Ki-67 antigens, using the standard avidin–biotin–peroxidase complex (ABC) method. In brief, after deparaffinization, endogenous peroxidase activity was quenched by immersing the sections for 20 min in absolute methanol containing 0.3% H_20_2. The sections were processed to unmask antigens by means of conventional microwave oven heating in 10 nM citric acid buffer (pH 6.0) and subsequent detergent treatment using polyoxyethylene sorbitan monolaurate (Tween 20) in phosphate-buffered saline for 30 min. The sections were then treated with normal serum for 20 min to reduce background staining, followed by treatment of primary antibodies at 4°C overnight. Anti-MDM2 monoclonal antibody (clone IF2; IgG2b class) was obtained from Oncogene Research Products (Cambridge, Mass.) and used at 1:40 dilution. Anti-Tp53 monoclonal antibody (clone DO-7; IgG2b class) was obtained from Dakopatts (Glostrup, Denmark) and used at 1:75 dilution. Anti-Ki-67 monoclonal antibody (clone MIB-1; IgG2b class) was obtained from Immunotech (Marseille, France) and used at 1:75 dilution. The sections were then treated for 1 h with biotinylated secondary antibodies (Vector Lab, Burlingame, Calif.), followed by treatment for 1 h using the ABC method (Vectastein ABC kit, Vector Lab). The reaction products were developed by immersing the sections in 3.3'-diaminobenzidine tetrahydrochloride solution containing 0.03% H_20_2. Nuclei were lightly counterstained with hematoxylin or methylgreen. No staining was obtained when nonimmune serum or phosphate-buffered saline was used instead of the primary antibodies.

Assessment of immunoreactivity

We assessed the three antigens in all tissue specimens. For MDM2, Tp53 and Ki67 analysis, only nuclear staining was regarded as positive. The percentage of tumor cells in each section exhibiting positive protein immunostaining was estimated by counting 500 cells in the five most representative areas. In both MDM2 and Tp53 immunostainings, cases of positive cells of 10% or more were regarded as positive, and cases of positive cells of less than 10% were categorized as negative. The cut-off points adopted to distinguish between positivity and negativity were defined as previously described [3, 27, 33, 42, 47, 48]. The Ki-67 labeling index was calculated by counting positive cells among 1000 nuclei.

Western blotting

Protein loads of 20 µg were applied onto a 10% polyacrylamide-sodium dodecyl sulfate (SDS) gel. The proteins were then transferred to nitrocellulose membrane (Amersham, San Diego, Calif.). The membrane was blocked with blocking solution [2.5% nonfat milk in phosphate-buffered saline (PBS)] for 20 min at room temperature and then incubated with antibody in the blocking solution. The antibodies to MDM2 and Tp53 were the same as those used in immunohistochemistry, and were used at dilutions of 1:50 and 1:125, respectively. After extensive washing with T-PBS (0.05% Tween20-PBS), the membrane was re-probed with anti-mouse IgG (class IgG/Fab', dilution 3:1000, MBL, Nagoya, Ja-

pan) conjugated with horseradish peroxidase in blocking solution for 2 h. Detection of signals was performed using the enhanced chemiluminescent technique and ECL kit (Amersham, Bucks, UK).

Correlations with clinicopathological features

Immunohistochemical results were correlated with histological grade, presence of vascular invasion and extrahepatic metastases, and UICC (*Union Internationale Contra la Cancrum*) stages for the 47 ICC patients [11]. All cases were adenocarcinomas according to the World Health Organization [12], and these were classified into well-, moderately- and poorly differentiated ICCs according to the Japan Liver Cancer Study Group [21]. The vascular invasion was assessed on elastica-van-Gieson stained sections and categorized into either present or absent.

Statistical analysis

Testing for associations among categorical variables was performed by means of χ^2 analysis. In the case of correlations between Ki-67 labeling index and several categorical variables, the Student's unpaired *t*-test was employed. A value of $P<0.05$ was considered to be statistically significant for all analyses.

Results

Immunohistology

The MDM2 and Tp53 immunoreactivities were heterogeneously observed in the nuclei of ICC cells (Fig. 1 and Fig. 2). The frequencies of MDM2 and Tp53 protein positivity in the tumor region were 18 of 47 (38%) and 27 of 47 (57%), respectively. MDM2 and Tp53 were not expressed in the non-tumorous areas. Fourteen cases were positive for both proteins (30%), and sixteen cases were negative for both proteins (34%). Ki-67 was also present in the nuclei of the tumor region and the Ki-67 positive cells ranged from 0.0 to 61.3. The mean and standard deviation (SD) of the Ki-67 labeling index was 14.1±14.0.

Western blotting

Western blotting showed that MDM2 was overexpressed in the tumor region. However, in the non-tumor region, only weak expression was detected. In the same case, the expression of Tp53 was also great in the tumor region, though no expression was detected in the non-tumorous part (Fig. 3).

Relationship with ICC clinicopathologic features

Correlations between MDM2 overexpression and ICC clinicopathological features are summarized in Table 1. Although the positive rate of MDM2 overexpression was highest in poorly differentiated ICC and lowest in well-differentiated ICC, there was no significant difference between the MDM2 overexpression and ICC tumor

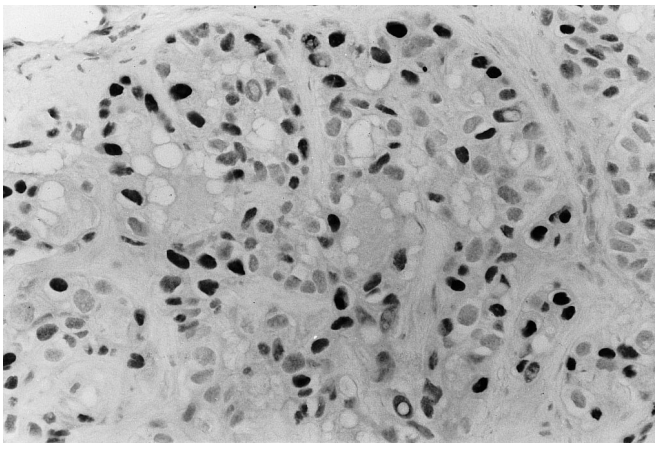

Fig. 1 Positive expression of MDM2 in cholangiocarcinoma. The expression was observed in the nuclei of cholangiocarcinoma cells. Immunostaining, ×400

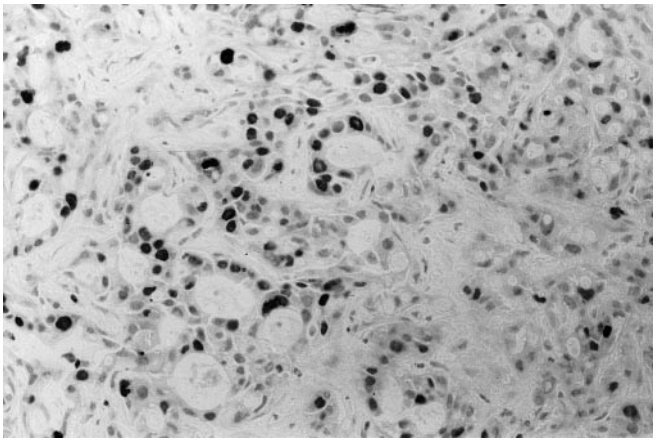

Fig. 2 Positive expression of Tp53 in cholangiocarcinoma. The expression was observed in the nuclei. Immunostaining, ×200

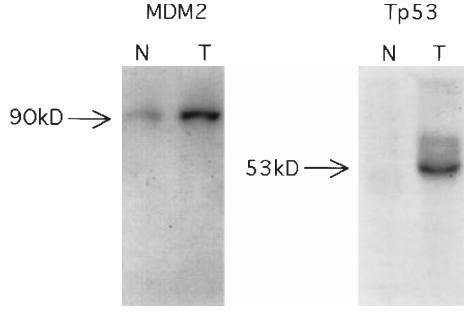

Fig. 3 Representative Western blot showing overexpression of MDM2 and Tp53 in the tumor region. *T* tumor region; *N* non-tumor region. In MDM2, a single distinct band of 90 kDa was present in the tumor region, while a single vague band of 90 kDa was present in the non-tumor region. In Tp53, a single band of 53 kDa is seen in the tumor region, but no band was present in the non-tumor region

Table 3 Relationship between overexpression of MDM2 and Tp53 in 47 cases of intrahepatic cholangiocarcinoma. Statistics: χ^2 test

		No. of cases examined		*P* value
		Tp53(+)	Tp53(–)	
MDM2 (+)	18 (38%)	14	4	<0.03
MDM2 (–)	29 (62%)	13	16	

Table 4 Relationship between overexpression of MDM2 and Ki-67 labeling in 47 cases of intrahepatic cholangiocarcinoma. *LI* labeling index. Statistics: student's *t*-test

		Ki-67LI (mean±SD)	*P* value
MDM2 (+)	18 (38%)	20.4±17.2	<0.03
MDM2 (–)	29 (62%)	10.3±10.0	

grade. The correlation between MDM2 overexpression and the presence of vascular invasion was also not significant. However, positivity of MDM2 overexpression significantly correlated with the presence of extrahepatic metastasis ($P<0.01$) and high tumor stage ($P<0.05$). The correlation between Tp53 expression and ICC tumor grade was not significant (Table 2). Other ICC clinicopathological features such as the presence of vascular invasion, extrahepatic metastasis, and tumor stage also did not significantly correlate with Tp53 expression. There also were no significant differences between Ki-67 labeling and ICC tumor grade, the presence of vascular invasion, extrahepatic metastasis, or tumor stage (data not shown).

Relationship between MDM2 overexpression and Tp53 overexpression and Ki-67 labeling index

Table 3 shows that MDM2 expression correlated with Tp53 expression ($P<0.03$). The means±SD of the Ki-67 labeling index in MDM2 positive and negative cases were 20.4±17.2 and 10.3±10.0, respectively. MDM2 expression also significantly correlated with the high Ki-67 labeling index (Table 4; $P<0.03$). However, there was no relationship between the Tp53 expression and high Ki-67 labeling index (data not shown).

Discussion

This is the first report of MDM2 expression in ICC. The frequency of MDM2 positivity in the tumor region was 18 of 47 (38%). The MDM2 immunoreactivities were observed in the nuclei of ICC cells, and no immunoreactivities of MDM2 were observed in non-tumorous cells. This finding was also observed using Western-blot analysis of MDM2. These findings suggest that intrahepatic bile ducts may newly express MDM2 during cholangiocarcinogenesis. In this study, MDM2 positivity significantly correlated with Tp53 positivity. This finding was consistent with previous studies of other carcinomas [17]. In breast carcinoma, Jiang et al. [17] immunohistochemically detected high MDM2-positive cases in 35% among 106 cancer specimens and in 72% of Tp53-positive cases, and also demonstrated that a higher MDM2 level directly correlated with the onset of lymph-node metastasis and shortened patient survival. In addition, Molina et al. [30] showed that overexpression of MDM2 and Tp53 in malignant fibrous histiocytoma was present in 48% and 40%, respectively. They also demonstrated that overexpression of MDM2 was present in 80% with Tp53 overexpression without *p53* gene mutations, and that co-expression of MDM2 and Tp53 significantly correlated with survival in the absence of gene alterations [30].

Overexpression of Tp53 was detected in 57% of our cases. This Tp53 overexpression was confirmed by means of Western blotting. In a recent series of ICC, overexpression of Tp53 ranged from 19.0% to 78.5% [35, 44, 50]. Rizzi et al. [44] found that ICC development in primary sclerosing cholangitis is commonly associated with overexpression p53 and that these occur at a late stage in the progression of the malignant process. These results suggest that the mutations of p53 were affected by the background of ICC, such as primary sclerosing cholangitis. In general, it has been considered that immunohistochemical detection of Tp53 represents mutations of the *p53* gene. In several cancer studies using the Tp53 monoclonal antibody clone DO7, such as bronchogenic and lung carcinoma or non-Hodgkin's lymphoma, *p53* gene mutations correlated almost always with Tp53 overexpression [8, 15, 51]. This is largely because mutant Tp53 has a much longer half-life than wild Tp53 [11, 23]. Moreover, in ICC, it has been indicated that among antibodies that can detect Tp53 in formalin-fixed, paraffin-embedded specimens, DO7 was the most useful antibody for detection of mutant Tp53, whereas PAb 1801, a clone of anti-Tp53 antibody, was unsuitable for it [50]. In some studies of other cancers, as for example esophageal adenocarcinoma, there was a discrepancy between *p53* gene mutations and Tp53 overexpression [49]. However, this immunohistochemical study [49] was performed using the p53 monoclonal antibody clone 1801, which recognizes a denaturation-resistant epitope located between amino acids 32 and 79 [6]. We therefore used the Tp53 monoclonal antibody clone DO7, which recognizes the epitope located between amino acids 35 and 45 and whose immunoreactivity apparently closely correlates with the presence of a *p53* gene mutation [50]. Therefore, our study suggests that 57% of the ICC cases have *p53* gene mutations.

To the best of our knowledge, in ICC, there have been no reports of the relationships between MDM2 and histological grade, clinicopathological features, tumor grade, Tp53 expression, or Ki-67 labeling index. The present study showed that MDM2 expression did

not correlate with ICC tumor grade and the presence of vascular invasion, indicating that ICC tumor grade and the presence of vascular invasion are independent of MDM2 expression status. However, our study showed that the positivity of MDM2 significantly correlated with the presence of extrahepatic metastasis and advanced tumor stage, suggesting that MDM2 is associated with the late stage of ICC progression. In addition, there was a significant association among Tp53 overexpression, Ki-67 labeling index, and MDM2 overexpression.

However, Tp53 overexpression did not significantly correlate with all investigated clinicopathological features, although Tp53 overexpression significantly correlated with MDM2 overexpression. The reason for this discrepancy may be caused by MDM2-induced degradation of Tp53. The inhibitory effects of MDM2 on the two central cell-cycle control pathways, Rb protein and p53, may induce further cholangiocarcinogenesis. Recently, it has been reported that interactions between MDM2 and Tp53 target Tp53 for rapid degradation [13, 21, 22]. In addition, it has been demonstrated that $p19^{Arf}$, a product of the *Ink4a* gene, inhibits MDM2 cotransformation activity, blocks MDM2-induced degradation of Tp53, and enhances p53-related activities such as transcription and apoptosis [40, 57]. ARF may also be activated by stress to cause MDM2 degradation and Tp53 accumulation [19].

In summary, our findings suggest that MDM2 overexpression is a rather late event in the progression of ICC, and may be associated with the occurrence of metastases.

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