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The expression of the *mdm2* gene may be related to the aberration of the *p53* gene in human hepatocellular carcinoma

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Abstract The relationship between *mdm2* gene expression and *p53* gene mutation in hepatocellular carcinoma (HCC) and their correlation with the invasiveness of the disease were investigated in this study. Either the expression level of the *mdm2* gene or the mutation rate of the *p53* gene was higher in HCC than in paratumor liver tissues. Studies on the relationship between *mdm2* and *p53* revealed that *mdm2* gene expression in HCC without *p53* mutation was higher than when there was *p53* mutation, while the *p53* mutation rate in HCC with *mdm2* overexpression was significantly lower than in HCC without *mdm2* overexpression. Among 23 HCC with invasion, *mdm2* gene overexpression was found in 6 patients while *p53* mutation was found in the other 11 patients, and only 1 patient was found to have both *mdm2* overexpression and *p53* mutation. These results indicated that either *mdm2* overexpression or *p53* mutation may be related to the invasiveness of HCC. Considering that an autoregulatory feedback loop between the *mdm2* and *p53* genes may exist, wild-type *P53* can induce the expression of *mdm2* via a *p53*-binding site in the *mdm2* gene, while MDM2 protein functions as a negative regulator of P53 protein. These results also suggest that *mdm2* may be related to the high invasiveness of HCC through inactivating the tumor-suppressor function of the *p53* gene.

Key words *mdm2* · *p53* · Hepatocellular carcinoma · Tumor invasiveness

Abbreviations RT-PCR reverse transcription-polymerase chain reaction · *HCC* hepatocellular carcinoma

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Introduction

Aberration of the *p53* gene is the most commonly detected abnormality in human cancers (Greenblatt et al. 1994). This aberration is achieved mainly through mutation of the *p53* gene itself, or through direct binding of cellular or viral proteins that either promote its degradation or block its ability to transactivate downstream genes involved in arresting cell growth or promoting cell death (Lane 1994). A considerable body of evidence has suggested that the tumor-suppressor activity of *p53* is critically dependent on its ability to transactivate these downstream genes (Pietenpol et al. 1994). Besides mutation, the function of the *p53* gene may also be compromised by the binding of proteins, such as the MDM2 protein (Barak et al. 1993; Cho et al. 1994; Kussie et al. 1996; Momand et al. 1992). MDM2 protein, a product of the *mdm2* gene, is notable in that it can inactivate *p53* by promoting the rapid degradation of P53 protein, inhibiting *p53*-mediated apoptosis and masking the transactivation domain of the P53 protein so that *p53* cannot interact with the transcriptional machinery, in fact, preventing *p53*-dependent apoptosis and transcription (Haupt et al. 1996, 1997; Kubbutat et al. 1997; Oliner et al. 1993). The aberration of *p53* through the interaction with *mdm2* is of particular interest from a therapeutic point of view, as the *p53* function could potentially be restored by disrupting this interaction. This may suggest a protocol of gene therapy for human hepatocellular carcinoma (HCC).

The gloomy prognosis of HCC is mainly related to its intrahepatic spread and metastasis, even in early stages, and a high recurrence or metastasis rate after resection for HCC patients. The 5-year recurrence rate for small HCC, even after radical resection, is still as high as 43.5%. Studies on the molecular basis for the high invasiveness of HCC suggest that the high recurrence rate is mainly associated with the carcinoma's biological characteristics (Tang 1996; Zhou et al. 1994). Previous work in our institute has revealed that alterations of

oncogenes, tumor-suppressor genes, metastasis-suppressor genes and growth factors, such as p21, p16 (CDKN2), mP53, nm23, Kai-1, transforming growth factor α, and epidermal growth factor receptor, are mainly related to intrahepatic spread or vascular invasion but show no relationship with tumor size and capsule status. It seems that the presence of multiple nodules and tumor embolus better reflect the high invasiveness of HCC than do tumor size and capsule status. In the present study, HCC with multiple nodules and/or tumor embolus was considered as invasive while HCC with a single nodule and without tumor embolus was deemed non-invasive.

The relationship between *mdm2* gene expression and *p53* gene mutation in HCC was investigated using the reverse transcription polymerase chain reaction (RT-PCR) and PCR combined with restriction endonucleases.

Materials and methods

Patient samples

Surgical samples of tumor and paratumor tissues were obtained from the livers of 42 patients with pathologically proven HCC in our institute from September 1995 to October 1996. There were 39 men and 3 women, their average age being 47.8 years. Tumor with a maximum diameter no more than 5 cm was referred to as small HCC, otherwise it was designated as large HCC.

Primers for PCR

1. The primers for amplifying *mdm2* and the β-actin gene were synthesized by ACGT company (Ottawa, Canada). The sequences of primers for *mdm2* were as follows: 5′- TTA TTA AAG TCT GTT GGT GCA-3′ (sense) and 5′-TGA AGG TTT CTC TTC CTG AAG-3′ (antisense). The sequences of the sense and antisense primers for the β-actin gene were 5′-CTA CAA TGA GCT GCG TGT GGC-3′ and 5′-CAG GTC CAG ACG CAG GAT GGC-3′ respectively.
2. The primer for amplifying the *p53* gene was synthesized by Cybersyn (Beijing, China), and had the following sequences: 5′-CTG GAG TCT TCC AGT GTG AT-3′ (sense) and 5′-GTT GGC TCT GAC TGT ACC AC-3′ (antisense).

RNA and DNA extraction

RNA

RNA was extracted by acid guanidinium thiocyanate/phenol/chloroform according to the literature (Chomczynski and Sacchi 1987). Briefly, 0.1 g tissues was minced and homogenized with 1 ml denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate with pH 7, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol), sequentially 0.1 ml 2 M sodium acetate (pH 4), 1 ml phenol (water-saturated) and 0.2 ml chloroform/isoamyl alcohol mixture (49:1) were added to the homogenate. The final suspension was shaken vigorously for 10 s and cooled in ice-water for 15 min. After centrifugation at 10 000 *g* for 20 min at 4°C, the aqueous phase, in which RNA was present, was mixed with 1 ml isopropanol, and then placed at −20°C for 1 h to precipitate RNA. Sedimentation at 10 000 *g* for 20 min was performed again and the resulting RNA pellet was dissolved in 0.3 ml denaturing solution, transferred into a 1.5-ml Eppendorf tube, and precipitated with 0.3 ml isopropanol at −20°C for 1 h. After centrifugation for 10 min at 4°C, the RNA pellet was dissolved in diethyl-pyrocarbonate(DEPC)-treated water.

DNA

DNA was extracted by Higuchi's method (Perkin Elmer/Cetus. Newsletter Amplification 1989 2:1). Briefly, 0.5 g tissues was minced and homogenized with 1 ml lysis buffer (0.32 sucrose, 10 mM TRIS/HCl at pH 7.5, 5 mM $MgCl_2$, 1% Triton X-100) and then centrifuged at 13 000 *g* for 30 s at room temperature. After centrifugation, the pellet was dissolved in 1 ml lysis buffer and centrifuged again at 13 000 *g* for 30 s. The above procedure was repeated three times. The pellet was then dissolved in 100 µl buffer containing 50 mM KCl, 10 mM TRIS/HCl at pH 8.3, 2.5 mM $MgCl_2$, 0.1 mg/ml white gelatin, 0.45% Nonidet P40, 0.45% Tween 20, and 10 mg/ml proteinase K, and digested at 55°C for 90 min. After the reaction had been terminated by incubation at 95°C for 10 min, the sample was centrifuged at 13 000 *g* for 2 s. The DNA in the aqueous phase was collected for further study.

RT-PCR

A 9-µl sample of total RNA, 20 U RNasin, 6 µl oligo (dT) (100 µg/ml), 6 µl dNTP (20 mM), 30 U avian myeloblastosis virus reverse transcriptase (Promega), reaction buffer and DEPC-treated water were added to a 0.5-ml tube and incubated at 42°C for 1 h. The PCR mixtures for amplifying *mdm2* and the β-actin gene each contained equal amounts of cDNA (10 µ1), sense and antisense primers (50 pmol each), Taq DNA polymerase (3 U), PCR reaction buffer and double-distilled H_2O. Samples were amplified through 35 consecutive cycles, each amplification cycle consisting of a denaturation at 94°C for 1 min, primer annealing at 57°C for 1 min, and extension at 73°C for 3 min. Cycles were preceded by incubation at 95°C for 5 min to ensure full denaturation of the target DNA, and were followed by an extra 7 min of incubation at 74°C after the final cycle to ensure full extension of the product. The PCR reactions were performed on a DNA thermal cycler (Perkin Elmer/Cetus Instruments, Norwalk, Conn.). The amplified fragments of *mdm2* and the β-actin gene were 336 bp and 240 bp respectively.

Analysis of *mdm2* gene expression

The PCR samples of *mdm2* were extracted with an equal volume of phenol/chloroform and precipitated from 0.1 mol/l of sodium acetate solution with 2.5 volumes of ethanol for restriction-enzyme analysis of the PCR product; restriction-enzyme digestion of the amplified product was performed with *Hin*dIII and *Rsa*I according to the manufacturer's suggested conditions.

After a separation on 2% agarose, the gel containing the RT-PCR product was put on an X-ray reading system and printed in "reverse" manner. The absorbance (A) and the area of fragment were read by an Image Analysis System (Appligene, Mitsubishi, Japan). The expression level of the *mdm2* gene was calculated as follows:

$$\textit{mdm2}\ \text{gene expression}\ (\%) = [\textit{mdm2}\ \text{fragment area} \times (A_{\text{fragment}} - A_{\text{background}})] / [\beta\text{-actin gene fragment area} \times (A_{\text{fragment}} - A_{\text{background}})].$$

Analysis of the *p53* mutation at codon 249

The PCR was carried out on 3 µl extracted DNA using 50 pmol each primer and 2.5 U Taq DNA polymerase in 10 × PCR buffer in a total volume of 50 µl for 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1.5 min. The amplified fragment was 110 bp. A5-µl aliquot of PCR product was used to analyze the mutation of the *p53* gene. The PCR products were digested at 37°C for 1 h with 10 U *Hae*III (Promega) in a total volume of 10 µl, then electrophoresed on 2% agarose gel. The wild-type *p53* would be cleaved

by *Hae*III at codon 249, giving products of 75 bp and 35 bp. Any changes at codon 249 would result in destruction of the *Hae*III site and an uncleaved product of 110 bp (Bressac et al. 1991).

Statistical analysis

The differences in the expression of *mdm2* and in the mutation rate of *p53* between different groups were analyzed for statistical significance using the *t*-test and χ^2-test, with the significance level set at $P < 0.05$.

Results

The amplification product of the *mdm2* gene contains the expected *Hin*dIII and *Rsa*I bands at the appropriate position. To verify their identity, *mdm2* amplification products were digested with *Hin*dIII and *Rsa*I restriction enzymes (Fig. 1).

The expected sizes of the amplified *mdm2* fragments following digestion with *Hin*dIII and *Rsa*I were 159 bp and 249 bp respectively (Bueso-Ramos et al. 1993). Figure 1 suggests that the 336 bp fragment was the *mdm2* amplified product.

The expression level of *mdm2* in HCC samples (52.0 ± 5.5%) was higher than that in paratumor tissues (26.2 ± 5.1%, $P < 0.01$). The expression levels of *mdm2* in different HCC groups are shown in Table 1. *mdm2* expression in HCC with invasion (62.6 ± 8.5%) was higher than that in HCC without invasion (39.1 ± 5.1%, $P < 0.05$). While no significant difference in *mdm2* expression was found between HCC with different capsule status or between large HCC (> 5 cm) and small HCC (≤5 cm).

No *p53* mutation at codon 249 was found in any of the paratumor liver tissues. Of the 42 tumors tested, 18 were found to have mutations of the *p53* gene at codon 249. *p53* mutation rates at codon 249 in different groups are shown in Table 2. The *p53* mutation rate was higher in HCC with tumor embolus than in that without tumor embolus. No significant difference in the mutational rate of *p53* was found between HCC with single and multiple nodules, small and large HCC, or HCC with and without capsule.

mdm2 gene expression in HCC without *p53* mutation (62.1 ± 8.4%) was higher than that with *p53* mutation (38.5 ± 4.8%, $P < 0.05$). As the mean level of *mdm2* gene expression in HCC with invasion was 62.6 ± 8.5%, this value was used to subdivide these patients into two groups according to the relative level of each patient: HCC with *mdm2* gene overexpression (higher than 62.6%) and HCC without *mdm2* gene overexpression (lower than 62.6%). The mutation rate of the *p53* gene at codon 249 in HCC with *mdm2* overexpression (1/10, 10%) was significantly lower than that in HCC without *mdm2* overexpression (17/32, 53.1% $P < 0.05$) (Fig. 2). Among these patients, HCC with both *mdm2* overexpression and *p53* mutation was found in only 1 patient, HCC with neither *mdm2* overexpression nor *p53* mutation in 15 patients, and HCC with either *mdm2* overexpression or *p53* mutation in 26 patients. Among 23 HCC with invasion, *mdm2* overexpression was found in 6 patients while *p53* mutation was found in the other 11 patients; only 1 patient was found to have both *mdm2* overexpression and *p53* mutation.

A total of 32 patients were followed-up over 1 year. Recurrence or metastasis within 1 year was found in 13

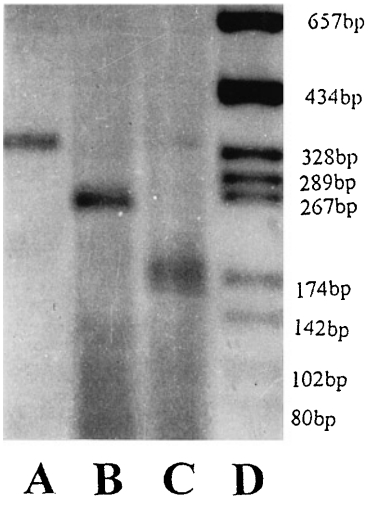

Fig. 1A–D Electrophoresis on 2% agarose gel of *mdm2* gene fragments produced by the *Hin*dIII/*Rsa*I restriction endonucleases. **A**. *mdm2* gene amplified fragment (336 bp), **B**. *Rsa*I digestion product (249 bp), **C**. *Hin*dIII digestion product (159 bp and 177 bp), **D**. markers

Table 1 *mdm2* gene expression in different hepatocellular carcinoma (*HCC*) groups

Group	No. of cases	*mdm2* gene expression, mean ± SE (%)	*P*
Invasive HCC	23	62.6 ± 8.5	< 0.05
Non-invasive HCC	19	39.1 ± 5.1	
Capsule			
Complete	15	53.6 ± 8.7	> 0.05
Incomplete	27	51.1 ± 6.6	
Small HCC (≤5 cm)	16	48.9 ± 5.5	> 0.05
Large HCC (> 5 cm)	26	53.9 ± 8.2	

Table 2 *p53* gene mutation in different HCC groups

Group	No. of cases	Mutation rate (%)	*P*
HCC with embolus	12	75.0	< 0.01
HCC without embolus	30	30.0	
Nodule			
Multiple	15	40	> 0.05
Single	27	44	
Capsule			
Complete	15	40	> 0.05
Incomplete	27	44	
Large HCC (> 5 cm)	25	44	> 0.05
Small HCC (≤5 cm)	17	41.8	

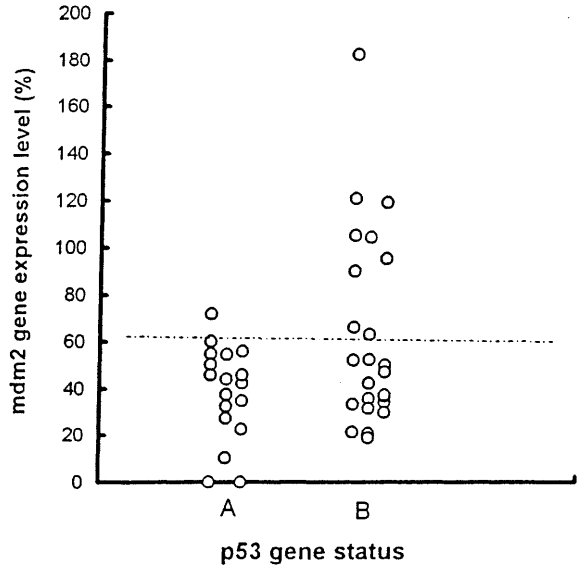

Fig. 2 The relationship between *mdm2* gene expression and *p53* gene mutation. *A* HCC with *p53* gene mutation. *B* HCC without *p53* gene mutation. —•— *mdm2* overexpression level (62.6%). The level of *mdm2* gene expression in HCC without *p53* mutation was higher than that with *p53* mutation. The mutation rate of the *p53* gene in HCC with *mdm2* overexpression was lower than that in HCC without *mdm2* overexpression

Table 3 *mdm2* gene expression in different prognostic groups

Group	No. of cases	*mdm2* gene expression, mean ± SE (%)	*P*
Recurrent	13	61.0 ± 8.5	< 0.05
Non-recurrent	19	40.6 ± 4.4	

patients. The other 19 patients were free of tumor. The expression level of the *mdm2* gene was higher in the group with recurrent disease than that in the non-recurrent group (Table 3).

Discussion

The *mdm2* gene maps to human chromosome 12 and represents an evolutionarily conserved gene with tumorigenic potential and a predicted role in mechanisms of cellular growth control (Fakharzadeh et al. 1991; Kondo et al. 1996; Momand and Zambetti 1997). It can form a negative-feedback loop that helps to limit the growth-suppressing activity of *p53*. In response to DNA damage, which leads to an increase in *p53*, wild-type *p53* can induce *mdm2* gene expression via a *P53*-binding site in the *mdm2* gene, while the induced MDM2 protein can result in a large and rapid reduction in P53 protein levels through enhanced proteasome-dependent degradation. The MDM2 protein forms a tight complex with both mutant and wild-type P53 protein and inhibits *p53*-mediated transactivation and apoptosis, thereby limiting the length or severity of the *p53*-mediated arrest following the DNA damage (Barak et al. 1993; Chen et al. 1994; Haupt et al. 1996, 1997; Jones et al. 1995; Kubbutat et al. 1997; Momand et al. 1992; de Oca Luna et al. 1995; Oliner et al. 1992, 1993; Perry et al. 1993). The crystal structure of the product of MDM2 and P53 protein interaction has recently been determined (Kussie et al. 1996). *mdm2* may also enhance the malignant phenotype in several cell lines, not only by conferring invasive activity but also by stimulating the expression of angiogenic growth factors (Fakharzadeh et al. 1991; Kondo et al. 1996). In this study, a higher expression of *mdm2* was found in HCC samples than in paratumor tissues. Considering that *mdm2* gene amplification and overexpression has also been found in other tumors, these results suggest that *mdm2* may relate to a malignant phenotype (Bueso-Ramos et al. 1993; Florenes et al. 1994; Ladanyi et al. 1993; Leach et al. 1993; Lianes et al. 1994; Lonardo et al. 1997; Marchetti et al. 1995; McCann et al. 1995; Oliner et al. 1992; Reifenberger et al. 1993; Watanabe et al. 1994). Further studies have indicated that the expression level of *mdm2* in invasive HCC is higher than that in non-invasive HCC, while no significant difference was found between HCC with and HCC without a capsule or between large and small HCC. These results suggest that the expression, especially the high-level expression, of *mdm2* is related to the high invasiveness of HCC. But the clinical implication of *mdm2* in human malignant tumor remains controversial (Lianes et al. 1994; Bueso-Ramos et al. 1993; Ladanyi et al. 1993; Watanabe et al. 1994). In the present study, high expression of the *mdm2* gene was apparently associated with recurrent HCC. This result further indicates that high expression of *mdm2* may be associated with more aggressive HCC.

Worldwide studies sequencing the entire coding region of *p53* have indicated that evaluations of mutations at codon 249 of *p53* are likely to have underestimated the prevalence of *p53* mutation in HCC in China (Greenblatt et al. 1994; Hsu et al. 1991). It has been reported that *p53* mutation may be associated with progression and invasiveness of HCC (Greenblatt et al. 1994; Hsu et al. 1993; Murakami et al. 1991; Nishida et al. 1993; Qin et al. 1995; Zhao et al. 1993; Zheng et al. 1996). In this study, the 42 HCC tested were compared according to the following characteristics of tumors: embolus, capsule status, number and size. The results revealed that 42.9% of HCC had *p53* mutation at codon 249, while no *p53* mutation was found in any of the paratumor tissues tested; *p53* mutation in tumors with embolus was more frequent than in those without embolus. No significant difference in the mutational rate of *p53* was found between HCC with single and multiple nodules, small and large HCC or between HCC with and without capsule. As vascular invasion usually reflects late-stage HCC, these results indicate that *p53* mutation might be related to the high invasiveness of HCC and occur in an advanced stage of the disease.

Since there exists an autoregulatory feedback loop between the *mdm2* and *p53* genes (Barak et al. 1993; Kussie et al. 1996; Meltzer 1994; Momand et al. 1992; Momand and Zambetti 1997; Oliner et al. 1993), these HCC patients were subdivided according to *p53* gene status and *mdm2* gene expression level, to study the relationship between *p53* mutation and *mdm2* expression. Expression of *mdm2* was found to be higher in HCC without *p53* gene mutation than in HCC with *p53* mutation, while the mutation rate of *p53* in HCC with *mdm2* gene overexpression was significantly lower than that in HCC without *mdm2* gene overexpression. Among 23 HCC with invasion, *mdm2* gene overexpression was found in 6 patients while *p53* mutation was found in the other 11 patients, and only 1 patient was found to have both *mdm2* overexpression and *p53* mutation. These results indicated that either *mdm2* overexpression or *p53* mutation may relate to the invasiveness of HCC. Tumors without *p53* alteration usually harbored a high expression level of the *mdm2* gene, while *p53* mutation occurred more frequently in HCC with low expression of *mdm2*, these results suggested that *mdm2* overexpression may be an alternative mechanism to the mutational inactivation of *p53* in tumorigenesis and that the major effect of *mdm2* gene overexpression is identical to that resulting from *p53* gene mutation (Barak et al. 1993; Leach et al. 1993; Meltzer 1994). Similar results have been found in other tumors (Leach et al. 1993; McCann et al. 1995; Reifenberger et al. 1993; Watanabe et al. 1994). The majority of cases having intact *p53* genes and *mdm2* gene overexpression indicate that *mdm2* gene expression is a response to *p53*. Through binding to P53 protein and inhibiting the normal function of the *p53* gene, *mdm2* might play an important role in inactivating *p53* in HCC. Interestingly, the overexpression of *mdm2* was independent of another *p53*-responsive gene ($p21^{WAF1}$), this result further indicates that the wild-type *p53* is at least inactivated at the transcription level by high amounts of MDM2 protein (Picksley et al. 1996). But nothing in cancer biology, including the relationship between *mdm2* expression and *p53* mutation, is ever quite as simple as it first appears. MDM2 protein can also bind to mutant P53 protein (Momand et al. 1992), and *mdm2* gene overexpression and *p53* mutation can occur in the same tumor patient (Meltzer 1994). On the other hand, the amplification unit that contains the *mdm2* gene is frequently quite large, and includes other genes related to the regulation of cell growth (Meltzer 1994). There also exists a correlation between estrogen receptor status and the *mdm2* mRNA level in human breast cancer (Sheikh et al. 1993). The *mdm2* gene could also negatively regulate the function of Rb protein and positively augment cell proliferation by stimulating the S-phase-inducing transcription factors E2F1/DP1 (Martin et al. 1995; Xiao et al. 1995). The anti-apoptotic effect of *mdm2* depends on the cellular context; it might block apoptosis in some cells, but not in others (Haupt et al. 1996). All this suggests these other molecular mechanisms might relate to HCC and the association of *mdm2* with HCC is worth further investigation.

In recent years, a number of landmark papers have been published that clearly establish *mdm2* as an oncogene promoting the tumor phenotype, at least in part, by antagonizing the tumor-suppressor function of wild-type *p53* (Meltzer 1994; Momand and Zambetti 1997). The present study indicated that the *mdm2* gene might be related to the high invasiveness of HCC through its inactivation of the tumor-suppressor function of *p53*. Retaining the normal function of *p53*, through disrupting the interaction between the *mdm2* and *p53* genes, may provide a new approach to gene therapy of HCC.

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