Cloning and Expression of a Developmentally Regulated Transcript MXR7 in Hepatocellular Carcinoma: Biological Significance and Temporospatial Distribution

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ABSTRACT

Using the differential display method to analyze mRNA expression in hepatocellular carcinoma (HCC) and nontumor livers, we cloned a full-length cDNA of 2263 bp, which was designated GTR2-2 and was identical with MXR7. The MXR7 mRNA was detected in 143 of 191 (74.8%) primary and recurrent HCCs taken from 154 patients but only in 5 (3.2%) nontumor livers. MXR7 mRNA was detected in one of two hepatoblastomas but not in hepatocellular adenoma, cholangiocarcinoma, or metastatic carcinomas to the liver. In human cancer of other anatomical sites, MXR7 mRNA was detected in low levels in one Wilms' tumor and in 4 of 40 gastric adenocarcinomas but not in several other types of cancer and 21 nonhepatocellular human tumor cell lines examined. MXR7 mRNA was expressed in high levels in the placenta, fetal liver, lung, and kidney, but it was undetectable in adult liver and was expressed in very low levels in adult lung and kidney. Our observations suggest that the MXR7 gene is regulated developmentally and expressed preferentially in HCC. To study its potential biological significance, we selected 113 patients who had unicentric primary HCC and had been followed for more than 4 years for further analysis. The MXR7 mRNA expression correlated closely with elevated serum α-fetoprotein (AFP) levels (88 versus 55%; P = 0.0001) and with expression of AFP mRNA (87 versus 55%; P = 0.005) and CD24 mRNA in HCC (80 versus 50%; P < 0.04), high tumor grade (76 versus 56%; P = 0.05), and tumor invasion (76 versus 55%; P < 0.05), but not with patient outcome. In HCC ≤3 cm, the frequency (77%) of MXR7 mRNA expression was significantly higher than that of elevated serum AFP (43%; P < 0.007) and AFP mRNA expression in HCC (41%; P < 0.004). Thus, MXR7 may serve as a sensitive early tumor marker for HCC and warrants more study to better understand its biological function.

INTRODUCTION

HCC is one of the most common cancers in Asia and Africa and is the leading fatal malignancy in Taiwan (1). Early detection with surgical resection is mandatory for prolonging life. Mass screening of small HCCs in the subclinical stage has been conducted successfully using a serum AFP screen (2). However, recent studies have shown that more than half of the HCCs that are 5 cm or smaller in size are missed if detection relies on serum AFP level alone (3–5). Hence, a more sensitive tumor biomarker is needed. During the multistep tumorigenesis of human cancer, multiple genetic abnormalities accumulate, and the tumor becomes more and more aggressive (6, 7). These genes, critical to tumor progression, remain to be identified. Tumor invasiveness is one of the most crucial prognostic histological factors for HCC (8, 9), but the molecular mechanisms of this are not clear. Although p53 mutation is an important molecular prognostic factor for HCC and is related closely to tumor invasiveness, p53 mutation is detected in only one-third of HCCs in Taiwan (10, 11). Little is known about the other crucial genes involved in tumorigenesis and progression in HCC. These critical genes may be expressed differentially in tumor and nontumor tissues, and theoretically can be detected using the DD technique (12–14). To explore these candidate genes in hepatocarcinogenesis, we conducted a DD screen of the mRNA expression patterns in paired HCC and nontumor liver samples. Using this method, we successfully cloned several candidate genes, including the CD24 gene that is overexpressed in HCC (15). In this report, we present our findings of a gene, designated GTR2-2 according to anchor and random primer sets. This gene, which is identical to MXR7 (16), was regulated developmentally in human liver, and was overexpressed preferentially in HCC, might serve as a sensitive early biomarker for HCC.

MATERIALS AND METHODS

Subjects. From January 1986 to December 1994, 439 patients with resected primary HCC had disease pathologically proven at National Taiwan University Hospital. The surgical specimens were immediately cut into small pieces, snap frozen in liquid nitrogen, and stored at −135°C in deep freezers. Parts of the tissues were also embedded in OCT embedding compound and stored at −70°C in deep freezers. For the analysis of tissue specificity and temporospatial tissue distribution of candidate cDNA sequences identified, human fetal tissues, neoplasms of other anatomical sites, and tumor cell lines were also studied. Fetal tissues were obtained from fetuses and newborns during autopsy. For adult tissues, we used commercially obtained multiple-tissue Northern blots containing approximately 2 μg of pure polyadenylated RNA from specific adult tissues (Clontech Laboratories, Inc., Palo Alto, CA).

Cell Lines. To determine the tumor specificity, the MXR7 mRNA expression was also examined in various types of human tumor cell lines: four derived from HCC, Hep 3B (ATCC HB-8064); ATCC, Rockville, MD), SK-Hep-1 (ATCC HTB-52), HuH7 (17), and HA22T/VGH (18); six from lung carcinoma, CL-1, CL-2, and CL-3 (19), PC-9, PC-13, and PC-14 (20); two from transitional cell carcinoma, NTUB1 (21) and T24 (ATCC HBT 4); one from breast adenocarcinoma, SK-BR-3 (ATCC HTB 30); one from renal cell carcinoma (ATCC CRL 1933); one from prostatic adenocarcinoma, PC-3 (ATCC CRL-1435); five from B cell lymphoma, Raji (ATCC CCL-86), IB4 (ATCC HB-10164), IBW4 (22), MC116 (ATCC CRL-1649), and LBF (23); three from T-cell lymphoma, HUT 102 (ATCC TIB-162), SR 786 (24), and H9 (ATCC HTB 176); and two from Hodgkin's lymphoma, L428 (25) and KM-H2 (26).

DD, Cloning, and Sequencing. The modified DD method (12, 13) was performed as described previously (15). Briefly, total RNA (2 μg) was reverse transcribed with 500 units of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD) in the presence of 3 μM T11GT (5'-TTTTTTTTTTTTTGT-3') as primer, 16 μM deoxynucleotide triphosphate, 16 μM DTT, 20 units of RNasin, and reverse transcription buffer in a 30-μl reaction volume for 60 min at 35°C. PCR was done in solution containing the anchoring primer T11GT and random primer R2 (5'-CTGATCCATG-3'). Bands showing differential expression of mRNA were cut out from the dried gel, eluted, reamplified, cloned, and sequenced as described previously (15).
Experimental Design. For the DD analysis, each reaction included 40 RNA samples taken from HCC, hepatoblastoma, hepatocellular adenoma, and their paired livers.

Histological Study. The tumor grade was simply divided into two groups, well (grades I and II) and poorly (grades III and IV) differentiated HCC, as described (10). HCCs were divided into noninvasive and invasive, as described in their paired livers.

Northern Blot Analysis. Total RNA was extracted as previously described (8, 9). Polyadenylated mRNA was purified by Oligotex-dT (Qiagen GmbH, Hilden, Germany) from the total RNA samples of HCC, in which specific mRNA of interest was detected on Northern blot. Double-stranded cDNA was synthesized and cloned into a Uni-ZAP XR vector using a ZAP-cDNA synthesis kit (Stratagene, La Jolla, CA). Approximately 2 × 10⁶ phages were screened. Positive plaques were selected for in vivo excision to generate the pBluescript phagemid from the Uni-ZAP vector, and the insert was sequenced as described previously (15).

5' RACE. The 5'-end sequences of the identified gene was determined by 5' RACE using a 5'-RACE kit (Clontech) according to the recommendations of the manufacturer as described (15, 27).

RNA in Situ Hybridization. For making riboprobes, the full-length cDNA of the candidate gene, designated GTR2-2 according to the anchor and random primers, was used as a template. For in vitro transcription and in situ hybridization, we used a DIG RNA Labeling Kit and DIG Nucleic Acid Detection Kit (Boehringer Mannheim Biochemica, Mannheim, Germany), respectively, according to the recommendations of the manufacturer. Briefly, paired 5 μm-thick deparaffinized sections mounted on acid-cleaned coated slides were hybridized with digoxigenin-labeled sense and antisense riboprobes, respectively, in hybridization solution for 16 h at 60°C. After being washed, the slides were incubated with antidigoxigenin antibody for 3 h at room temperature, colored in nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate solution, and then either counterstained with methyl green or mounted with no counterstain.

Controls. Positive controls included HCC and fetal tissues that expressed MXR7 (GTR2-2) mRNA. Negative controls included adult liver, HCC, and fetal tissues that had no MXR7 (GTR2-2) mRNA expression, and hybridization with a sense riboprobe.

Statistics. We used the Student's t test for statistical analysis. A P of less than 0.05 was considered significant.

RESULTS

Cloning and Sequencing of GTR2-2 (MXR7) cDNA. In DD analysis, we identified a candidate band that was expressed preferentially in HCC (Fig. 1). The full-length cDNA of 2263 bp was cloned from a HCC-derived cDNA library, and the 5'-end sequence was identified by 5' RACE. The cDNA sequence that was designated GTR2-2 (GenBank accession no. LA47176) according to the anchor and random primer sets had an identity of 100% in a 2225-bp overlap with human MXR7 cDNA (16) identified in human gastric adenocarcinoma. Hence, MXR7 was used.

Expression of MXR7 mRNA in Hepatic Neoplasms, Tumors of Other Anatomical Sites, and Tumor Cell Lines. Using GTR2-2 cDNA as a probe, we could hybridize a 2.3-kb mRNA in HCC on Northern blot but not in nontumor liver (Fig. 2). Among 191 primary or recurrent tumors taken from 154 patients, MXR7 mRNA was detected in 143 tumors (74.8%) and was absent in 48 (25.1%). In contrast, MXR7 mRNA was detected in low levels in only five nontumor (three cirrhotic and two noncirrhotic) livers (3.2%), three of which had intrahepatic portal vein tumor spread and one of which had distant satellite nodules. Of the tumors with MXR7 mRNA expression, two tumors (1.2%) had an abnormally smaller transcript (Fig. 2). In other types of hepatic tumors, MXR7 mRNA was found in one of two hepatoblastomas but not in hepatocellular adenoma or cholangiocarcinoma (Table 1).

Among tumors of other anatomical sites, MXR7 mRNA was de-
AFP mRNA expression in HCC (87 versus 55%; P = 0.005). MXR7 mRNA was detected in 2 of 4 HCC cell lines (Fig. 4) but not carcinomas to the liver or in several types of cancer, including cases (51%). The tumor size was 5 cm (small HCC) in 63 patients invasive patients, anti-hepatitis C virus was detected in sera in 20 (74%) of 27 cases examined. The serum AFP level was above 320 ng/ml in 58 patients with primary or recurrent HCC, or both, but it was expressed in low levels in only three cirrhotic and two noncirrhotic adult livers (3.2%), four of which had either portal vein tumor spread or distant satellite tumor nodules. To better understand the potential biological significance of MXR7 mRNA, we investigated its temporospatial expression in hu-

**Table 1 Expression of MXR7 mRNA in malignant and benign hepatic tumors, metastatic carcinomas, tumors of other anatomical sites, and tumor cell lines**

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>MXR7 mRNA expression (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCC</td>
<td>143 (74.8)</td>
</tr>
<tr>
<td>Hepatoblastoma</td>
<td>2</td>
</tr>
<tr>
<td>Hepatocellular adenoma</td>
<td>1</td>
</tr>
<tr>
<td>Cholangiocarcinoma</td>
<td>6</td>
</tr>
<tr>
<td>Metastatic colorectal carcinoma</td>
<td>5</td>
</tr>
<tr>
<td>Gastric adenocarcinoma</td>
<td>40</td>
</tr>
<tr>
<td>Wilms’ tumor</td>
<td>1</td>
</tr>
<tr>
<td>Soft-tissue sarcoma</td>
<td>6</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>6</td>
</tr>
<tr>
<td>Cell line</td>
<td></td>
</tr>
<tr>
<td>HCC</td>
<td>4</td>
</tr>
<tr>
<td>Non-HCC</td>
<td>21</td>
</tr>
</tbody>
</table>

*These included 114 cases with unicentric and 21 cases with multicentric primary HCC, 5 cases with transhepatic arterial embolization before tumor resection, and 14 cases with both primary and one or more recurrent HCCs.

**DISCUSSION**

To identify a potential early biomarker for HCC, we compared several important biomarkers in HCCs ≤3 cm in size. We found that MXR7 mRNA was expressed in 77% (or 27 of 35), significantly higher than serum AFP elevation (43%, or 15 of 35 cases; P < 0.007) and AFP mRNA expression (41%, or 12 of 29 cases; P < 0.004), but the frequency of MXR7 mRNA expression was similar to that (80%, or 20 of 25 cases) of CD24 mRNA, which is an early biomarker for HCC (15). The expression of MXR7 mRNA did not correlate with patient outcome; regardless of tumor size or p53 mutation (data not shown), both are important prognostic factors for HCC (8–11).

Using a mRNA DD (12–15), we successfully cloned a full-length cDNA of 2263 bp of a gene that was expressed preferentially in HCC. The cDNA sequence was designated GTR2-2 according to the anchor and random primers (European Molecular Biology Laboratory/Gen-Bank/DNA Data Bank of Japan accession no. LA7176). The cDNA sequence was identical to that of MXR7 (16) identified in gastric carcinoma. The MXR7 mRNA appeared as a major transcript of 2.3 kb and encoded a putative protein of 580 amino acids containing a potential secretory signal sequence but no apparent transmembrane domain. A smaller transcript of about 1.1 kb was identified in two tumors. Thus far, little is known about MXR7 gene expression in human tissues and cancers. We found that the MXR7 mRNA was expressed in 143 of 191 (74.8%) tumors taken from 154 patients with primary or recurrent HCC, or both, but it was expressed in low levels in only three cirrhotic and two noncirrhotic adult livers (3.2%), four of which had either portal vein tumor spread or distant satellite tumor nodules. To better understand the potential biological significance of MXR7 mRNA, we investigated its temporospatial expression in hu-

![Fig. 3. Northern blot analysis of MXR7 in four different types of human cancer. Lane 1, cholangiocarcinoma; Lane 2, seminoma of the testis; Lane 3, neuroblasto-
ma of the adrenal gland; Lane 4, Wilms’ tumor of the kidney; Lane 5, HCC as positive control. 28S rRNAs were used for evaluating the quality and quantity of RNA loading.](https://cancerres.aacrjournals.org/article/57/8/5181/3)

![Fig. 4. Northern blot analysis of MXR7 in four human HCC cell lines. Lane 1, Hep 3B; Lane 2, Hep 7; Lane 3, HA22T/VGH; Lane 4, SK-Hep-1. 28S and 18S rRNAs were used for evaluating the quality and quantity of RNA loading.](https://cancerres.aacrjournals.org/article/57/8/5181/4)
Fig. 5. Northern blot analysis of MXR7 in non-hepatocellular human tumor cell lines. None of the tumor cell lines derived from B- (Lane 1, Raji cell; Lane 2, MC116; Lane 3, IB4; Lane 7, IBW4; and Lane 9, LBF) and T-cell lymphomas (Lane 5, SR786, and Lane 8, H9), Hodgkin's lymphoma (Lane 4, L428, and Lane 6, KM-H2), renal cell carcinoma (Lane 11, 1933), prostatic adenocarcinoma (Lane 12, PC3), transitional cell carcinoma of urinary bladder (Lane 13, NTUB1), or lung adenocarcinoma (Lane 15, CL1) showed a positive signal for GTR2-2 (MXR7) mRNA. Positive controls: Lane 10, HCC; Lane 14, Hep 3B. 28S and 18S rRNAs were used for evaluating the quality and quantity of RNA loading.

**Table 2: Distribution of MXR7 mRNA in adult and fetal human tissues**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Heart</th>
<th>Brain</th>
<th>Lung</th>
<th>Liver</th>
<th>SkM</th>
<th>Kid</th>
<th>Pan</th>
<th>Adr</th>
<th>Spl</th>
<th>Thym</th>
<th>Pros</th>
<th>Tes</th>
<th>Ov</th>
<th>Sml</th>
<th>Col</th>
<th>Leuk</th>
<th>Pla</th>
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</thead>
<tbody>
<tr>
<td>Fetus</td>
<td></td>
<td></td>
<td>2+2</td>
<td>+2</td>
<td>NT</td>
<td>NT</td>
<td>-</td>
<td>+1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>+3</td>
</tr>
<tr>
<td>Adult</td>
<td>+1</td>
<td>+1</td>
<td>-</td>
<td>+1</td>
<td>tr</td>
<td>+1</td>
<td>NT</td>
<td>tr</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+1</td>
<td>tr</td>
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</tr>
</tbody>
</table>

* Signal intensity: -, absent; tr, trace; +1, weak; +2, moderate; +3, strong.
* Pla, placenta; SkM, skeletal muscle; Kid, kidney; Adr, Adrenal gland; Spl, spleen; Thym, thymus; Pros, prostate; Tes, testis; Ov, ovary; Sml, small intestine; Col, colon; Leuk, peripheral leukocytes; NT, not tested.
* Tissues were obtained during autopsy at National Taiwan University Hospital.
* Premade multiple adult tissue RNA blots were obtained commercially (Clontech Laboratories).
Expression of MXR7 in HCC

Fig. 7. RNA in situ analysis of MXR7 in HCC. Using an antisense riboprobe (A and C) derived from a full-length MXR7 cDNA, a positive signal was detected in HCC (T) but not in the nontumor liver (L). Higher magnification (C) disclosed a positive signal in the cytoplasm of almost all of the tumor cells (arrows). A sense probe gave negative results (B and D). Magnification: A and B, ×52; C and D, ×510.

Man fetal and adult tissues and several types of cancer and cancer cell lines other than HCC. MXR7 mRNA was expressed in abundance in the placenta and several fetal tissues, particularly liver, kidney, and lung, but the levels became undetectable in adult liver and decreased dramatically in adult kidney and lung. By RNA in situ hybridization analysis, we could detect MXR7 mRNA in the tumor cells of HCC, fetal hepatocytes, pulmonary bronchial, renal tubular, and gastric mucosal epithelial cells. These findings indicate that MXR7 is a developmentally regulated gene. This suggestion is supported partly by the fact that the cDNA sequence of MXR7 is 86% homologous to the cDNA of OCI-5, which is regulated developmentally in rat intestine and highly expressed in rat fetal intestine (31). The high homology between MXR7 and OCI-5 suggests that they may be closely related and highly conserved through evolution.

In hepatic tumors other than HCC, we found that MXR7 mRNA expression was detected in one of two hepatoblastomas, but not in three hepatocellular adenomas, six cholangiocarcinomas, or five metastatic colorectal carcinomas to the liver. Among several types of cancer of other anatomical sites, MXR7 mRNA was detected in low levels in 4 of 40 (10%) gastric adenocarcinomas and in one Wilms’ tumor but not in several other types of cancer, including soft-tissue sarcomas. Among 21 nonhepatocellular human tumor cell lines, including non-Hodgkin’s lymphoma and Hodgkin’s disease, MXR7 mRNA was detected in none. These findings indicate that MXR7 mRNA is overexpressed preferentially in HCC and can serve as a tumor biomarker for HCC.

To explore the potential biological role of MXR7 mRNA expression, we selected 113 patients who had unicentric primary HCC and had been followed for more than 4 years after tumor resection or until death for further analysis. Of these, the expression of MXR7 mRNA was found in 81 cases (71.7%) and correlated positively with elevation of AFP levels in serum (88 versus 55%; P = 0.0001) and expression of AFP mRNA (87 versus 55%; P = 0.005) and CD24 mRNA (80 versus 50%; P < 0.04) in HCC. The high level of MXR7 mRNA expression in fetal livers, shutoff in adults, and resurgence in HCC resemble AFP gene expression. These findings suggest that MXR7 expression is also an oncodevelopmental gene. Although an elevated serum AFP level is regarded as a tumor marker for HCC and has been used successfully for screening of HCC (2), our observations confirm that fewer than half of the patients with small-tumor HCC have an elevated serum AFP level (3—5). The significantly higher frequency of MXR7 mRNA expression (77%) than that of elevated serum AFP (43%; P < 0.007) and AFP mRNA expression (55%; P = 0.005) in HCCs ≤3 cm suggests that MXR7 mRNA expression is a more sensitive early tumor marker for HCC.
occurs more often in invasive HCC and is an important unfavorable factor in both large and small HCCs (8, 9). Although p53 mutation facilitates tumor cell growth and contributes to tumor progression, molecular mechanisms for the function of the MXR7 gene are not well understood (10, 11). Hence, more studies are warranted to better understand the biological role of MXR7 in HCC.

**REFERENCES**

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