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ABSTRACT

We have identified a novel human malignancy-associated gene (MAG) expressed in various malignant tumors including glioblastomas and hepatocellular carcinomas (HCCs) and in tumor preexisting conditions such as hepatitis C virus- and hepatitis B virus-induced liver cirrhosis. The expression of MAG was characterized using reverse transcription-PCR (RT-PCR), rapid amplification of cDNA ends PCR, RNA dot blotting, RNase protection assay, and Northern blot analysis. Rapid amplification of cDNA ends PCR yielded a 536-bp MAG fragment in HCC, macroregenerative liver nodules with dysplasia, and liver cirrhosis but not in normal liver or placenta. By RT-PCR, MAG expression was not found in 12 different normal tissues but found in 46 of 51 (90%) premalignant and malignant tissues of various sites. Embryonic liver and brain were positive for MAG expression together with tumors from the same organs, but the corresponding normal adult tissues were negative. By RNase protection assay, MAG mRNA was expressed in the HepG2 liver tumor cell line and in an ovarian carcinoma but not in normal liver. The estimated transcript size from Northern blot analysis was 8.8 kb. This novel gene may play a role in the progression of premalignant conditions and in the development of HCC and other cancers.

INTRODUCTION

Cellular proto-oncogenes, tumor suppressor genes (anti-oncogenes), and DNA mismatch repair mutations are generally considered to be key molecular genetic biomarkers of carcinogenesis (1, 2). However, only a few genes have been shown to be useful for clinical tumor diagnosis and prognosis. Concurrent loss of p53 function may contribute to the clinical aggressiveness of pancreatic carcinomas. Similarly, loss or mutation of a new candidate tumor suppressor, DPC4 (deleted in pancreatic carcinoma locus 4), is reported in pancreatic cancer (3). In breast cancer, HER-2/neu amplification has greater prognostic value than other identified factors (4). Overexpression of HER-2/neu rarely occurs in the absence of gene amplification in breast cancer (~3% of cases; Ref. 5). Prostate-specific antigen expression is a marker for prostate cancer (6). In liver cancer, α-fetoprotein, an embryonic protein, is expressed in 26-53% of cases, and loss of c-myc expression and high β2 microglobulin expression is a marker for prostate cancer (6). In liver cancer, a-foetoprotein, an embryonic protein, is expressed in 26-53% of cases, and loss of c-myc expression and high β2 microglobulin expression is a marker for prostate cancer (6).

MATERIALS AND METHODS

Materials. Fresh-frozen human tissues (a total of 69 samples), which were not used for diagnosis, were obtained from the Department of Pathology and Laboratory Medicine, Cedars-Sinai Medical Center. Cultures of human tumor hepatocyte cell lines, HepG2 and Hep3B (American Type Culture Collection, Rockville Pike, MD), were propagated according to the American Type Culture Collection protocol. Total RNA from human adult brain, embryonic liver, and embryonic brain was purchased from Clontech Laboratories (Palo Alto, CA).

Gene Sequencing. Sequencing was carried out using Sanger’s method (13) with a Sequenase kit (United States Biochemical, Cleveland, OH). [32P]dATP was obtained from Amersham (Arlington Heights, IL). Sequencing gels were run on an ABI Standard Sequencer STS 45 (New Haven, CT). cDNA fragments subcloned in pGEM 3Zf(+) plasmids were prepared for sequencing according to the Promega Biotech (Madison, WI) commercial protocol.

Preparation of Tissue mRNA. Total RNA (50–100 μg) was isolated according to a published method (14), with modification (15), using TRIzol reagent (Life Technologies, Inc., Gaithersburg, MD). Poly(A)-containing RNA was prepared by purification of RNA on oligo(dT) cellulose, using the Poly(A) Pure mRNA isolation kit (Ambion, Inc.). DNase/RNase treatment of the RNA was performed by digesting total RNA for 1 h at 37°C, using 20 units of either DNase or RNase. Both enzymes were purchased from Ambion, Inc. (Austin, TX). RT-PCR was carried out as described previously (15, 16). From the novel gene sequence, several sets of primers were designed for fragments of different sizes: (a) 104-bp fragment: forward primer 5′-AAGTTATATTAGAATGGACATCTGTTGCTC-3′; and reverse primer 5′-CTTGATGCAAAGTCGGTGCTC-3′; and (b) 127-bp fragment: forward primer 5′-GGACTTGATTCTCTGTCCATTCGACGTC-3′ and reverse primer 5′-CTCTCTCTATATATAAGGCTTCTGTA-3′. Routine negative controls (without reverse transcriptase and a water control) and a positive kit control were included in each reaction. 3′ RACE PCR was carried out using a 5′/3′ RACE kit (Boehringer Mannheim, Indianapolis, IN). 3′ RACE takes advantage of the natural poly(A)+ tail of mRNAs as a priming site for PCR amplification. First-strand cDNA synthesis is initiated at the poly(A)+ tail of mRNA using the oligo(dT) anchor primer. After converting mRNA into cDNA, the amplification is then directly performed without a further purification step using the PCR oligo(dT) anchor primer, 5′-GACACCCGGTATCGATGTCGACTTTTCTTTTTT-3′, and a MAG-specific primer, 5′-CCAGCTTGGCAGCAGTTACTAT-3′.

RNA Dot Blot Analysis. The 536-bp 3′RACE PCR fragment was cloned into the Smal site of pGEM 3Zf(+) plasmid (Promega Biotech) and used as a random primed probe. Total tissue RNA was transferred to nylon membranes (Schleicher & Schuell, Keene, NH) using a Bio-Rad (Richmond, VA) dot-blotting apparatus. RNA was fixed onto the membrane by short-wave UV irradiation using Stratagene (Stratagene, La Jolla, CA) for 40 min. Membranes were prehybridized for 4 h at 42°C in 50% deionized formamide, 7% SDS, 10% BSA, 1 mM EDTA, and 0.2 M sodium phosphate (pH 7.2). The 536-bp cDNA MAG probe was radiolabeled by random primer oligolabeling in the presence of [α-32P]dCTP. A nick-translated probe (a total of 5 × 106 cpm) was used in the hybridization solution. Membranes were hybridized at 42°C for 20 h.

Transcription Reaction. A 276-bp fragment of MAG gene cloned in pGEM 3Zf(+) was used to prepare the antisense probe. Antisense 276-bp probe was obtained by digesting the 536-bp probe, according to the restriction map. Two μg of DNA template, 10× transcription buffer with DTT, a mixture of premalignant and malignant tissues but not in normal tissues. We have designated this gene MAG.
of nucleotides containing 1 µl of each (ATP, CTP, and GTP, all 10 mM), 5 µl of 10 mM solution of labeled [32P]UTP (a total of 5 × 10^6 cpm), 2 µl of T7 RNA polymerase + RNase inhibitor (Ambion, Inc.), in a total volume of 20 µl were used. After incubation, 1 µl of RNase-free DNase I (2 U/µl) was added to the reaction for 15 min at 37°C (17).

**RPA.** RPA II kit (Ambion, Inc.) was used according to the supplied protocol. Briefly, poly(A)+ pure RNA (1 µg for each sample) was hybridized with 2 × 10^6 cpm of [32P]UTP-labeled antisense probe and incubated overnight at 42°C. Equivalent amounts of total yeast RNA and yeast tRNA were set up as controls for the probes to be used. Two hundred µl of concentrated RNAse A/RNase T1 mixture in RNAse digestion buffer (1:50) was added to all experimental tubes and to one tube of each pair of yeast RNA of control tubes. To the remaining yeast RNA control tubes, only 200 µl of RNase digestion buffer without RNase were added and incubated for 20 min at 37°C. Samples were analyzed on 5% polyacrylamide/1 X Tris Borate EDTA buffer gels with T7 bp, and 390 bp were analyzed on 5% polyacrylamide/1 X Tris Borate EDTA buffer gels with T7 bp. Proteins in the gel lanes (Fig. 1) were visualized by silver staining (22). As a control, we used β-actin cDNA (Life Technologies, Inc.) labeled as described for MAG. After hybridization with a MAG-specific probe, the same blot was stripped and reprobed for ß-actin (18). All samples were positive for β-actin. Hybridization and washing were done exactly as for MAG treatment. Membranes were autoradiographed at −70°C overnight.

**RESULTS**

When studying the expression of the c-met proto-oncogene in normal and neoplastic human liver by RT-PCR, we used primers specific for c-met (16). In cirrhotic and HCC livers, but not in normal liver, an extra PCR band of approximately 150 bp was observed consistently. This band might represent either an alternatively spliced variant of c-met or a novel human gene amplified under low stringency conditions. This band was cut from the gel and sequenced. Only a short stretch of sequence homology with c-met was revealed in the 154-bp sequenced RT-PCR fragment. Specific primers for RT-PCR analysis of the novel gene were then designed, in the area on a sequence map between c-met primers.

Using 3'RACE PCR analysis, we obtained a larger cDNA fragment of 536 bp, partially overlapping the original 154-bp fragment. 3'RACE PCR yielded a 536-bp product in hepatitis C virus cirrhotic liver, HCC, and liver macrogeneitive nodules with dysplasia, but not in normal placenta (not shown) or normal adult donor liver in the same experiment (Fig. 1). 3'RACE PCR was performed twice, and the results were identical. The complete sequence of the obtained cDNA (569 bp), based on the alignment of 154- and 536-bp fragments, is shown in Fig. 2. Sequence analysis revealed that the entire 154-bp sequence contained an open reading frame (Fig. 2). The data suggest that the respective gene may be coding for a real protein.

**RT-PCR with specific primers for the 104-bp sequence within this fragment (outside of a short c-met homology region) was used to study the expression of the putative novel gene in 18 human fresh-frozen samples of explanted human liver tissues by RT-PCR. These samples included four normal livers, one fulminant hepatic failure liver, seven cirrhotic livers (two, with small HCC nodules), and six HCCs. No expression of the novel gene was detected in the four normal livers and one fulminant hepatic failure liver. In contrast, the expression of this gene was detected in all cirrhotic livers and in five of six HCCs. No expression of the novel gene was detected in all cirrhotic livers and in five of six HCCs. Another set of novel gene-specific primers for a 127-bp product was designed to test normal adult and fetal brain and liver and four glioblastomas. All glioblastomas (one grade III and three grade IV) were positive for the novel gene expression, but normal adult brain was negative (Fig. 3b). Fetal liver and brain were compared for MAG expression with normal adult liver and brain and with tumors of the same organs. Normal adult liver and brain were negative for MAG expression, but embryonic and malignant liver and brain tissues did express MAG (Fig. 3c).

In the second series of RT-PCR studies of normal and diseased human tissues, the expression of the novel gene was found in the vast majority of malignant and premalignant tissues but not in normal or nonmalignant (fulminant hepatic failure) liver tissues (Table 1). As a necessary control, we confirmed that the RT-PCR product obtained in tumors other than liver tumors was indeed a novel gene fragment. The corresponding PCR band from several randomly chosen tumors was sequenced, and the sequence was found to be identical to that of the 104-bp novel gene fragment. On the basis of the expression of the novel gene in malignant tumors but not in normal human tissues, we have decided to refer to the novel gene as MAG.

The 536-bp 3'RACE PCR fragment was used as a probe for dot blot analysis of RNA extracted from 4 normal tissues and 16 tumor and premalignant tissues (Fig. 4). Positive signals were detected in 9 of 16 malignant and premalignant conditions [HCC, macrogeneitive liver nodule with dysplasia, metastasis of an endometrial carcinoma, two hepatitis C liver cirrhoses, carcinomas of the lung, colon, ovary (one of two), and a colon carcinoma surrounding tissue]. However,
To determine the MAG transcript size, Northern analysis was used to screen several tissues for MAG expression with the 536-bp cDNA fragment as a probe (Fig. 8). Positive signal was observed in cirrhotic liver, where samples in Lanes 12 and 13 had small nodules of HCC. Lane M, 100-bp DNA ladder.

To get more conclusive evidence, in the third approach the RPA method was used on poly(A)+ mRNA from normal adult donor liver, an ovarian carcinoma, a colon carcinoma, and two cultured human liver tumor cell lines, HepG2 and Hep3B (Fig. 7). The probe was protected from RNase digestion (positive MAG mRNA expression) in ovarian carcinoma and HepG2 cells, in contrast to normal liver, colon cancer, and Hep3B cells, which were also all negative for MAG expression by RT-PCR (not shown). The RPA results were thus in complete agreement with RT-PCR data on the same mRNA samples.

Table 1  Expression of novel MAG gene in normal and diseased human tissues revealed by RT-PCR

<table>
<thead>
<tr>
<th>Type of tissue</th>
<th>No. of samples with MAG expression</th>
<th>Total number of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td></td>
<td></td>
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<tr>
<td>Liver</td>
<td>0</td>
<td>4</td>
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<tr>
<td>Gallbladder</td>
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<td>Placenta</td>
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</tr>
<tr>
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<td>1</td>
</tr>
<tr>
<td>Kidney</td>
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<td>1</td>
</tr>
<tr>
<td>Brain</td>
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<td>1</td>
</tr>
<tr>
<td>Spleen</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Nonmalignant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fulminant hepatic failure liver</td>
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<td>6</td>
</tr>
<tr>
<td>Tumor and premalignant</td>
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<td></td>
</tr>
<tr>
<td>Cirrhotic liver (6 HBV + 10 HCV)*</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>Liver macroregenerative nodule*</td>
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<td>2</td>
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<td>10</td>
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<td>1</td>
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<tr>
<td>Glioblastoma (grades III and IV)</td>
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</tr>
<tr>
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<td>1</td>
</tr>
<tr>
<td>Breast carcinoma</td>
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<td>2</td>
</tr>
<tr>
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<td>1</td>
</tr>
<tr>
<td>Renal carcinoma</td>
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<td>1</td>
</tr>
<tr>
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<td>4</td>
</tr>
<tr>
<td>Tissue surrounding colon carcinoma*</td>
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<td>Prostate carcinoma</td>
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<td>1</td>
</tr>
<tr>
<td>Endometrial carcinoma</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Metastasis of endometrial carcinoma*</td>
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<td>1</td>
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</table>

* HBV, hepatitis B virus; HCV, hepatitis C virus.
* One sample with dysplasia.
* From a patient with MAG-positive primary tumor.

Fig. 4. RNA dot blot analysis of MAG expression in normal and malignant human tissues. Lane A: 1, normal liver; 2, normal placenta; 3–4, normal gall bladders; 5, HCC; 6, macroregenerative liver nodule with dysplasia from the same patient; 7, endometrial carcinoma; and 8, metastasis of endometrial carcinoma from another patient. Lane B: 1–2, HCV cirrhotic livers; 3, lung carcinoma; 4, colon carcinoma; 5–6, ovarian carcinomas; 7, prostate carcinoma; and 8, uterine carcinoma. Lane C: 1–2, 3–8, blank; 3, colon carcinoma; and 4, tissue surrounding colon carcinoma from the same patient. Although the dots 6–8 from Lane B are negative, a more sensitive RT-PCR revealed MAG expression in all these cases (not shown).
In this study, a partial characterization is reported of a novel gene expressed in various human tumors and premalignant conditions but not in normal tissues. This expression pattern prompted us to tentatively name this gene MAG.

Initially, a 154-bp MAG fragment was obtained in RT-PCR experiments involving c-met proto-oncogene with which it shares a short region of homology. Subsequent 3'RACE PCR allowed us to extend the MAG sequence to 569 bp. Recently, a genomic sequence of the human ERCC2 gene and of two adjacent genes, C and KLC2 (referred to as the ERCC2 gene cluster), appeared in GenBank (19).3 Sequence comparison of MAG and this gene cluster revealed a minus strand stretch in the ERCC2 cluster, which has 95% sequence similarity with the 569-bp MAG fragment. This stretch is located within a 3' 12-kb region of the ERCC2 gene cluster 54-kb genomic sequence. Because the sequence homologous to MAG lies outside of all three genes in this cluster, MAG most probably represents a novel gene. It should be noted that 3'RACE PCR yielded a continuous sequence of the new gene from the initial fragment down to the poly(A) tail that suggested that we were dealing with mRNA for this gene (MAG).

It then became necessary to establish more rigorously that MAG was really expressed at the mRNA level and that it was not artifically amplified from residual DNA present in our RNA preparations. To this end, several methods were used that all confirmed the existence of MAG mRNA: (a) RNA samples were pretreated with RNase-free DNase and DNase-free RNase before RT-PCR. After DNase treatment of RNA samples, a usual MAG band was obtained, but after RNase treatment, no band could be revealed; (b) Northern blot analysis of several tissues revealed a single band of about 8.8 kb in tumor but not in normal tissue, in full agreement with the data obtained by RT-PCR; and (c) RPA analysis of mRNA from normal and tumor tissues and cultures showed specific protection of the MAG sequence from RNase digestion in tumor but not in normal tissues. Taken together, these data strongly suggest that MAG is indeed expressed at the mRNA level and that this expression is specific to tumors and premalignant conditions.

To extend the 569-bp cDNA MAG fragment in the 5' prime direction, we performed 5'RACE PCR. However, this attempt was unsuccessful. This may be due to a large transcript size of MAG that precluded its full-length amplification by 5'RACE PCR.

Although the molecular characterization of the novel MAG gene is not complete, its interesting expression pattern has been revealed. RT-PCR analysis of large number of samples from human tumors and tumor preexisting conditions (such as liver cirrhosis and macroregenerative nodules) has shown a striking difference between lack of expression in normal tissues and positive expression in most premalignant and malignant tissues.

Importantly, MAG expression originally found in liver tissue was detected not only in liver cirrhosis, premalignant macroregenerative nodules with dysplasia, and HCC, but in various other human malig-
nancies including glioblastomas and carcinomas of the lung, breast, colon, kidney, prostate, endometrium, ovary, and uterus. This is similar to other examples in which there is a specific gene expression in tumors (20). For instance, prostate-specific antigen expression is a reliable biomarker for prostate cancer. However, the expression of this antigen is also found in breast cancer (21). Moreover, the HER-2/neu gene, a biomarker for breast cancer, was amplified in nondiploid prostate cancers but not in diploid prostate cancers (22).

Our study was focused primarily on liver and brain diseases, such as cirrhosis, HCC, and glioblastomas. To determine whether MAG belongs to the group of oncofetal genes (23), we have also analyzed its expression in normal embryonic human liver and brain. The results demonstrate that MAG is expressed in fetal and malignant liver and brain tissues but is not expressed in corresponding adult normal tissues. Our data regarding MAG expression in fetal and malignant tissues versus adult normal tissues agree with the data on the expression of embryonic genes in some tumors. Relevant examples include α-fetoprotein expression in both embryonic liver and HCC (24, 25), and midkine, a neurotrophic and angiogenic growth factor, which is produced in fetal and malignant astrocytes but not in normal adult astrocytes (26). It has been shown that human fetal brain and malignant glial tumors express monocyte chemoattractant protein-1 in vivo and in vitro, but normal adult brain does not (27). Analysis of 30 liver and 5 brain disease cases for MAG expression has indicated its potential importance in liver and brain neoplasia. The absence of MAG expression in different normal tissues and its presence in various malignancies supports the hypothesis that this novel gene may play an important role in the process of malignant transformation.

Most recently, we have established a tissue culture system to study MAG expression and its regulation. This system involves hepatic HepG2 and Hep3B tumor cell lines. By RT-PCR and RPA, MAG expression was detected only in the HepG2 cell line. Its expression could be inhibited in HepG2 cells by factors important for liver and other tissue growth, hepatocyte growth factor (10 ng/ml, 48 h), and transforming growth factor β1 (1.5 ng/ml, 48 h). Thus, the MAG expressing HepG2 cell line can be used to study MAG regulation of expression, to clone its full-length cdNA, and to analyze properties of putative MAG-encoded protein. The MAG nonexpressing Hep3B cell line may be a good candidate for future gene transduction experiments, once the complete MAG sequence is obtained.

In summary, we have partially characterized a novel human gene (MAG) that is expressed at the mRNA level in malignant and premalignant tissues of various sites but not in different normal tissues. It may be a new member of the group of oncofetal genes (23) because it is also expressed in fetal liver and brain, and may play a role in embryogenesis and tumor development.

ACKNOWLEDGMENTS

We thank Drs. Donald Brown and Konstantin Spirin for valuable comments, Dr. Karen McKeown for expert editorial assistance, and Yvette Middleton for excellent technical contribution with tissue culture.

Unpublished data.

REFERENCES


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Novel Human Malignancy-associated Gene (MAG) Expressed in Various Tumors and in Some Tumor Preexisting Conditions

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