M6P/IGF2R gene is mutated in human hepatocellular carcinomas with loss of heterozygosity

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The mannose 6-phosphate/insulin-like growth factor II receptor (M6P/IGF2R) functions in the intracellular trafficking of lysosomal enzymes, the activation of the potent growth inhibitor, transforming growth factor β, and the degradation of IGF2 (ref. 1), a mitogen often overproduced in tumours3-6. We have recently shown that 70% of human hepatocellular tumours have loss of heterozygosity (LOH) at the M6P/IGF2R locus7 which maps to chromosome 8q26–q27 (ref. 8). Using a coarsen screen, we have now identified point mutations in the remaining allele of 25% of human hepatocellular carcinomas (HCCs) with LOH. These mutations give rise to truncated receptor protein and significant amino acid substitutions, and provide evidence that the M6P/IGF2R gene functions as a tumour suppressor in human liver carcinogenesis.

Using total RNA from human HCCs exhibiting LOH at the M6P/IGF2R locus7, we screened for corresponding mutations in the remaining allele using Ambion's Mismatch Detect™ assay. In one HCC, reverse transcription polymerase chain reaction (RT-PCR) amplification of the region flanked by the primers M6P-6092Forward and M6P-7143Reverse produced one band with normal template and two bands with tumour template (Fig. 1a). The lower tumour (LT) band migrated with the normal band whereas the upper tumour (UT) band was approximately 200 base pairs (bp) larger, suggesting a possible insertion. When the gel purified normal, UT and LT bands were reamplified and used as templates for mismatch detection, the UT band produced two cleavage products suggesting a mismatch whereas the LT band was indistinguishable from the normal control (Fig. 1b). Direct sequencing of the UT band revealed a 192-bp insert in the M6P/IGF2R message (Fig. 1c). The presence of this insert was confirmed using an RNase protection assay (data not shown).

We amplified the corresponding region on genomic DNA from normal and tumour templates. The expected amplon size for this region was 230 bp and 422 bp for normal and mutant tumour cDNA templates, respectively. However, an amplon of ~1200 bp was observed (data not shown), suggesting an insert size of ~970 bp. Direct sequencing revealed that the insert observed in tumour cDNA was the 5' end of an intron that corresponds to

Fig. 2 Mutant intron splicing in a HCC with LOH. a, Sequence for the 5' end of the intron (intron 40 based on the mouse gene) from genomic DNA. Exon and intron sequences are shown in upper and lower case, respectively. The C–G→A–T transversion is highlighted with a black box. Box A is the normally used 5' splice site and Box B is the mutant 5' splice site in the tumour. The numbers indicate the position of the intron within the full length M6P/IGF2R mRNA transcript. b, Direct sequencing (coding strand) of intron DNA amplified from genomic DNA showing the C–G→A–T transversion (white triangle) in tumour. The mutant and surrounding normal DNAs were sequenced in both directions (data not shown). Contaminating normal DNA is observed at the mutant locus as a co-migrating band. c, Proposed model for intron splicing in tumour, 5' and 3' splice sites are shown boxed. The gua and aga dinucleotides present on the 5' and 3' boundaries of introns, respectively, are highlighted in bold type10,11. The C→A–T transversion is highlighted with a black box. Mutant splicing results in the 5' end of this intron remaining in mature M6P/IGF2R message.

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intron 40 in mice (Fig. 2a) (ref. 9). Furthermore, a comparison of the homology between the normal and tumour intron sequences revealed a C→A point mutation in the tumour (Fig. 2a, b).

The C→A transversion observed in tumour genomic DNA appears to create an alternative 5′ splice site within this intron (Fig. 2a, box B) whose sequence is more closely related to the consensus sequence (AGGTAAGT) for 5′ splice sites than the normally used 5′ splice site (Fig. 2a, box A)10.11. Therefore, the 5′ end of this intron becomes incorporated into the M6P/IGF2R message, causing a dramatic effect on translation since the first codon after that for Ser 2023 becomes the stop codon, TGA (Figs 1c, 2c). Truncation of the M6P/IGF2R protein at this position prevents synthesis of both the transmembrane domain and the lysosomal trafficking signals of this receptor.1 This would result not only in the secretion of the mutant M6P/IGF2R but also of lysosomal enzymes.12

Immunohistochemical staining of this HCC was performed using a polyclonal antibody against the M6P/IGF2R protein (Fig. 3a). As predicted from the C→A intron mutation, the primary staining for the M6P/IGF2R was observed extracellularly. In contrast, in an HCC without LOH and lacking this C→A transversion, strong intracellular staining for the M6P/IGF2R was observed (Fig. 3b) consistent with the observation that normally 90% of the receptors are within the cell. A second identical C→A intron mutation was also observed in a HCC without LOH.

A G→T transversion was also identified in a second HCC with LOH (Fig. 4a, b). This mutation, which is in a region corresponding to exon 31 of the mouse gene,13 results in a Gly1449Val substitution (Fig. 4d). Using the Chou-Fasman algorithm, this amino acid alteration is predicted to increase β-sheeting (data not shown). Additionally, Gly 1449 is conserved among human, bovine, rat and mouse (Fig. 4d), providing further support for a significant functional role for this amino acid. A second identical G→T transversion was observed in a HCC without LOH. Further analysis of this region revealed a third mutation, a G→A transition in an HCC with LOH (Fig. 4c). The G→A transition results in a Gly1464Glu substitution (Fig. 4d), and appears to alter protein secondary structure by decreasing β-sheeting (data not shown). The M6P/IGF2R protein contains 15 repeat domains; repeats 3 and 9 have been implicated in the binding of M6P residues14,15 and repeat 11 in the binding of IGF2 (ref. 16). Both the G→T transversion and the G→A transition are located in repeat 10, a region closely associated with ligand binding.

Thus, by using large (1,000–1,500 bp) contiguous cDNA fragments to screen for mismatches in the M6P/IGF2R gene, we have identified mutations in 25% (3/12) of HCCs with LOH and in one without LOH. Three different mutations have been detected, and both the G→T and G→A transversions were found in two separate liver tumours. A finer mismatch analysis of this locus with overlapping cDNA templates will be required to determine whether other mutations are present in liver tumours.

In mice the M6P/IGF2R gene is maternally imprinted17 whereas in humans imprinting appears to be a polymorphic trait.18-20 Thus, mice and any humans who are imprinted may have an increased susceptibility to liver tumours since only one mutation would be required to render the gene inactive. Since the M6P/IGF2R is normally present in the circulation12, mutant receptors in the plasma may potentially be helpful in liver tumour detection. Furthermore, mutated receptors on the plasma membrane of liver tumour cells might provide a surface antigen for the targeting of both therapeutic and diagnostic agents to liver tumours.

In conclusion, this is the first report to demonstrate that
in HCCs with LOH at the M6P/PG2R locus, there are accompanying mutations in the remaining allele that result in truncated receptor protein and major amino acid substitutions. These findings support the postulate that the M6P/PG2R functions as a tumour suppressor in human liver carcinogenesis.

Methods

RT-PCR analysis. First-strand cDNA synthesis was performed as follows: 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl2, 10 mM DTT, 6.5 mM dNTP mix, 1 μM reverse primer (see below), 2–5 μg total RNA, and 200 units M-MLV reverse transcriptase (GIBCO BRL) in a total volume of 20 μL. RNA and H2O were mixed and heated to 65°C for 5 min and then chilled on ice prior to the addition of the other ingredients. The final mixture was incubated at 37°C for 60 min and then at 45°C for 10 min. After one extraction with a mixture of phenol/chloroform/isoamyl alcohol (25:24:1 v/v/v), excess reverse primer was removed using a CHROMA SPIN-100 DNA purification kit (Clontech). 1 μL of the eluate was used in PCR as follows: 10 mM Tris-HCl pH 8.3 (1×), 50 mM KCl, 1 mM MgCl2, 200 μM dNTP mix, 200 μM forward and reverse primers (see below), 1 μL RT product, and 2.5 units Taq DNA polymerase (Perkin Elmer) in a total volume of 100 μL. Hot-start PCR was carried out under the following conditions: 1 min denaturing at 94°C, 1 min annealing at 55°C, and 1 min extension at 72°C for 35 cycles with an additional 5 min extension for cycle 35 on a DNA Thermal Cycler (Perkin Elmer).

Because the fidelity of Taq DNA polymerase is such that errors can be introduced during the PCR process, a number of precautions were taken. Mutant templates were amplified in two or more independent PCR and templates were generated from both RNA and genomic DNA. In all cases the corresponding normal template was amplified in parallel with the tumour template, and the tumour mutations were confirmed by direct sequencing in both directions.

Mismatch Detect™ assay. Sequence differences between normal and tumour cDNA templates were identified according to the manufacturer’s instructions (Ambion Inc.). Utilizing the T7 and SP6 phage promoters, both forward and reverse transcripts were synthesized, respectively, from the normal and tumour cDNA templates. The normal 17 transcript was hybridized to tumour SP6 transcript and vice versa. Hybridization of T7 and SP6 transcripts from normal cDNA provided control. Following RNAse A digestion, the products were analysed on an agarose gel. Mutations were identified either by the appearance of new bands or an increase in band intensity relative to that in the control.

Identification of the Cg-J-Act transversion. The following primers were used in RT-PCR. RT primer was M6P-7166R (5'-GAACCTTCTTCAACAGAAGTG-3') and C gag-1531R (5'-ACCACAGGGAGGAGG-3'). First round primers were M6P-692F (5'-AACAGCAATTGTGCCTCGTC-3') and M6P-7143R (5'-ACAGTTTCTCATGGGGTGTC-3'). The UT, ET and normal bands were gel purified prior to second round amplification and sequenced. The primers used for amplification were M6P-692F and M6P-6031R (5'-GTCGGTCAACTCCAGACG-3') and SP6-M6P-689R (5'-TCACATTGGTGACACATTAGGATTTGTCGCGG-3'). Gel purification of this DNA fragment, direct sequencing was done using M6P-692F (5'-CAGCATCTCCGCGATGACCGC-3') and M6P-6031R (5'-CAGTTTCTCCAGCTCCAGAGG-3').

Identification of the GC-3 transversion and the GC-J-Act transition. The following primers were used in RT-PCR. RT primer was M6P-5231R (5'-CGGAGCGGAGGATTAGAGGTCCGC-3'). First round primers were M6P-8312F (5'-TGGAGAGCCACAGCTTGAGG-3') and M6P-5418R (5'-ACACAGCAGGGATGAAAGG-3'). Second round primers were 5'-CTG-6296F (5'-GATGAGCCAGCATTACGAGGCGAGCAGTTCG-3') and 5'-SP6-M6P-5436R (5'-TTACATTGAGTACACATTAGGCACTTTGAG-3'). Genomic DNA was amplified using the primer pair M6P-4051F (5'-CGGCGGCTCCATCGCAAGGCCG-3') and M6P-4598R (5'-CAGCTTCTCAGGCTCGGAG-3'). PCR sequencing were directly sequenced using M6P-4411F (5'-CCAGAAGCCGCGCCTGCTG-3') and M6P-4598F (5'-TGGCGGACCAGTACAGGCTG-3'). Reagents for the broad mismatch screen of the M6P/PG2R message are available upon request.

Immunohistochemical staining for the M6P/PG2R. Frozen sections, 6 μm in thickness, were fixed in 100% ethanol and immunoperoxidase stained with diaminobenzidine as described. All sections were immunohistochemically stained in an identical manner and at the same time. Immunostaining, using immune rabbit IgG was used as a negative control on serial sections. The sections were counterstained with haematoxylin.

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