

M6P/IGF2R Tumor Suppressor Gene Mutated in Hepatocellular Carcinomas in Japan

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Mannose 6-phosphate/insulin-like growth factor II receptor (M6P/IGF2R) tumor suppressor– gene mutation is an early event in human hepatocellular carcinoma (HCC) formation in the United States, but its role in hepatocarcinogenesis in Japan is unclear. We therefore determined M6P/IGF2R mutation frequency in HCCs from patients who resided in the southern, central, and northern regions of Japan. Ten single nucleotide polymorphisms were used to identify HCCs and dysplastic liver nodules with M6P/IGF2R loss of heterozygosity. The retained allele in these tumors was also assessed for point mutations and deletions in the M6P/IGF2R ligand binding domains by direct sequencing of polymerase chain reaction (PCR) amplified DNA products. Fifty-eight percent (54 of 93) of the patients were heterozygous at the M6P/IGF2R locus, and 67% (43 of 64) of the HCCs and 75% (3 of 4) of the dysplastic nodules had loss of heterozygosity. The remaining allele in 21% of the HCCs contained either M6P/IGF2R missense mutations or deletions, whereas such mutations were not found in the dysplastic lesions. In conclusion, M6P/IGF2R is mutated in HCCs from throughout Japan with a frequency similar to that in the United States. Loss of heterozygosity in dysplastic liver nodules provides additional evidence that M6P/IGF2R haploid insufficiency is an early event in human hepatocarcinogenesis. (HEPATOLOGY 2002;35:1153-1163.)

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world, and it is especially prevalent in Southeast Asia and sub-Saharan Africa. The marked geographical variation in HCC frequency has resulted in the identification of hepatitis B virus (HBV), hepatitis C virus (HCV), aflatoxin B₁, and alcohol as etiologic risk factors for the disease. The incidence of HCC is increasing in many countries, par-

ticularly in regions in which HCV infection is more common than HBV infection.¹ Although the incidence of HCCs in the United States has almost doubled since the 1980s, it is still much greater in Japan where the liver cancer mortality rate is now more than 23 deaths per 10⁵ persons per year.²

Genome-wide microsatellite analyses and comparative genomic hybridizations show that genetic alterations in HCCs occur at a number of chromosomal locations, including 1p, 1q, 2q, 4q, 6q, 8p, 8q, 9p, 9q, 10q, 13q, 14q, 16p, 16q, 17p, and 19 (for review see Buendia³ and Grisham⁴). Some of these loci contain genes already known to function as tumor suppressors. *p53* loss of heterozygosity, at chromosomal location 17p13, generally occurs late in HCC transformation with an allelic loss frequency ranging from 25% to 60%.^{3,4} In regions of Africa and the southeast coast of Asia, in which chronic HBV infection is endemic and aflatoxin B₁ exposure is high, codon 249 of *p53* is also a hot spot for mutation in HCCs.^{5,6} Thus, *p53*-dependent intracellular pathways are often disrupted in HCCs. The Rb pathway in HCCs is also dysregulated by *p16^{INK4A}* (9p21) promoter hypermethylation.⁷ In contrast, the *Wnt* signaling pathway is reactivated in about 40% of HCCs through mutations in *β-catenin* and *axin* tumor suppressor genes that reside at

Abbreviations: HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; M6P/IGF2R, mannose 6-phosphate/insulin-like growth factor II receptor; PCR, polymerase chain reaction; polyG, poly deoxyguanosine.

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chromosomal locations 3p21 and 16p13.3, respectively.^{3,4,8} Nevertheless, to date only a few tumor suppressor genes have been clearly identified to function in liver carcinogenesis even though loss of heterozygosity occurs frequently at a number of chromosomal locations.

The *mannose 6-phosphate/insulin-like growth factor II receptor (M6P/IGF2R)* gene maps to 6q25-27,⁹ a chromosomal location predicted to contain a liver tumor suppressor gene.^{3,4} It encodes for a receptor that functions in intracellular lysosomal enzyme trafficking, transforming growth factor β activation, and IGF2 degradation.¹⁰ Granzyme B internalization by the M6P/IGF2R is also required for cytotoxic T cells to induce apoptosis in cells targeted for death, resulting in this receptor being referred to as a "death receptor."¹¹ M6P/IGF2R deficiency during embryogenesis results in cardiac abnormalities, cleft palate, fetal overgrowth, and perinatal lethality.^{10,12} Furthermore, large offspring syndrome in cloned animals is frequently associated with epigenetic changes in *M6P/IGF2R* imprint regulation that result in decreased gene expression.¹³ The *M6P/IGF2R* therefore plays a crucial role in regulating mammalian fetal growth and development.

The *M6P/IGF2R* is also mechanistically involved in the genesis of human cancer.¹⁴⁻¹⁹ *M6P/IGF2R* loss of heterozygosity coupled with intragenic loss-of-function mutations in the remaining allele is a common event in human breast, liver, and lung cancers.¹⁴⁻¹⁷ Tumor cell growth is also inhibited when *M6P/IGF2R* expression is restored to normal, whereas it is increased when gene expression is reduced.²⁰⁻²² Thus, both mutational and functional studies show that the *M6P/IGF2R* meets the criteria required to be classified as a tumor suppressor gene.²³

Although *M6P/IGF2R* is mutated in 61% of HCCs in patients from the United States,¹⁴⁻¹⁷ its role in hepatocarcinogenesis in Japan is unclear^{24,25} because Wada et al.²⁴ reported that HCCs in Japanese patients are not mutated at the *M6P/IGF2R* locus. We therefore determined the *M6P/IGF2R* mutation frequency in HCCs from patients residing in 3 geographically distinct regions of Japan (*i.e.*, Fukuoka, southern Japan; Toyama, central Japan; and Iwate, northern Japan). Our results show that *M6P/IGF2R* mutation occurs in HCCs with a similar frequency in American and Japanese patients irrespective of their residential location in Japan.

Patients and Methods

Patient Population

Paraffin-embedded HCC tissue sections were obtained from 56 patients that resided in the southern region of Japan (Fukuoka University Chikushi Hospital, Fukuoka,

Japan), 18 patients that resided in the central region of Japan (Toyama Medical and Pharmaceutical University, Toyama, Japan), and 19 patients that resided in the northern region of Japan (Iwate Medical University; Iwate, Japan). These 93 patients were used to determine the *M6P/IGF2R* mutational status of 110 HCCs and 19 dysplastic nodules obtained from 95 needle biopsy specimens, 25 surgical resections, 6 needle necropsies, and 3 autopsies. All patients had chronic liver disease resulting from HCV infection (75 patients), HBV infection (11 patients), autoimmune hepatitis (1 patient), or alcohol-induced hepatitis (1 patient); the cause of liver damage was unknown in 5 patients. The size and grade of the HCCs were obtained without knowledge of their *M6P/IGF2R* mutational status.

Laboratory Methods

Paraffin-Embedded Tissue Microdissection. Microdissection of tumor and surrounding normal liver tissue from 7- μ m histology sections was performed as previously described.²⁶ Briefly, paraffin-embedded sections were deparaffinized in xylene (2 \times 5 minutes), exposed for 2 minutes to graded ethanol washes (*i.e.*, 100%, 95%, 70%, and 50% ethanol) and rehydrated in H₂O before staining. The tissue sections were then stained for 30 seconds with 2% (wt/vol) methylene blue, and rinsed in H₂O before allowing them to air dry. Tumor and surrounding normal tissues (>50 cells) were carefully microdissected using a serial section stained with hematoxylin-eosin for comparison; the normal tissue used for genotyping was always connective tissue. The dissected tissues were then placed in 75 μ L of Tris-ethylenediamine-tetraacetic acid buffer (10 mmol/L Tris-HCl, pH 8.0 at 25°C and 0.5 mmol/L ethylenediaminetetraacetic acid, pH 8.0 at 25°C) containing 5 μ L of 20 mg/mL proteinase K (Boehringer Mannheim, Indianapolis, IN). This mixture was incubated at 52°C for 3 hours and then at 85°C for 10 minutes. Polymerase chain reaction (PCR) analysis was conducted using 5 μ L of this mixture, as described below.

Determination of *M6P/IGF2R* Loss of Heterozygosity. Ten identified single nucleotide polymorphisms were used to assess the HCCs for *M6P/IGF2R* loss of heterozygosity (Table 1). The heterozygosity rate and allelic frequency for these single nucleotide polymorphisms in both the Japanese and American population are published.^{27,28} These polymorphisms were used for loss of heterozygosity determination following 2 rounds of semi-nested PCR amplification; exon specific forward and reverse primers are provided in Table 1. HCCs were classified as having *M6P/IGF2R* allelic loss if loss of heterozygosity was detected at 1 or more polymorphic sites.

Table 1. M6P/IGF2R Loss of Heterozygosity Analysis of Paraffin-Embedded Tissues

Position	Nucleotide*	Genotype	Amplicon Size (bp)	F1 Primer (5' to 3')	R1 Primer (5' to 3')	Nested Primer (5' to 3')†
Exon 6	901	C/G	91	CACCAGGCGTTTGTATGTTGG	gaaagtcaggctcctgctggag	R2: aaacgcccaacagcatcaggag
Intron 7	X8-22	T/G	139	gtggaaaatctgcattaagctgc	ctctgtggcaggcactactcag	R2: gcaggcactcagtaatccac
Exon 9	1197	A/G	123	actaagtaagactgtaattcttaatacc	CTGTATTTCAGTTTCTCCACAG	F2: aatactattcatataaaacaagcctc
Exon 12	1737	G/A	111	TATTTGTACAGAGTGTGCAGG	ctaactcattccaactggatgcc	R2: gaaaagcatcacctagatcttc
Exon 16	2286	A/G	187	GAAGCTTTCATATTATGAT	caccacagggcatgagtatcctc	R2: gcattgatgatcctcaggagc
Intron 22	X23-42	T/C	105	ctgcactgtgcttggggctgc	ccctgagtaaacatcatcattg	R2: tcattgcaaaacaggggagc
Exon 34	5002	G/A	86	GTCCCCTTGTCCCTCCAAATC	GAGATGAGCATGGGCCTA	F2: TCCAAATCCGGCCTGAGC
Exon 40	6206	A/G	118	GGGTGTGATGTGACATTTGAGTGG	GCGGGTGGACTGGGAAGGC	F2: GGAGTGCAAATTCGTCCAGAAAC
Intron 46	X47-107	A/G	103	gtcattcagaatgtggggaaatgc	gtggtcagcttactatcact	R2: tgtagagagggcatgggagc
Intron 46	X47-5	T/A	72	atgccctctctacactggagta	CCTACAGCAAGTGGTCAGCTTAC	R2: GTGGTCAGCTTACTATCACTG

*Nucleotide position is based on Morgan et al.²⁹ Intronic polymorphisms are numbered relative to nearest exon.

†Nested primers F2 and R2 should be paired with R1 and F1, respectively.

The exons containing these polymorphisms were PCR amplified from genomic DNA using 1.5 U Platinum Taq DNA polymerase (Life Technologies, Baltimore, MD), 15 pmol primers, 1.5 mmol/L MgCl₂, and 100 μmol/L dNTPs in a 30-μL PCR reaction volume (94°C × 15 seconds, 55°C × 5 seconds, and 72°C × 30 seconds for 35 cycles). Direct sequencing of gel-purified PCR products was performed according to the manufacturer's protocol (Thermo Sequence; USB Corporation, Cleveland, OH). All HCCs with *M6P/IGF2R* loss of heterozygosity were confirmed by PCR amplification 2 or more independent times.

Determination of M6P/IGF2R Mutations. HCCs with *M6P/IGF2R* loss of heterozygosity were also screened for point mutations and deletions in the remaining allele by direct sequencing of purified PCR products. Because the *M6P/IGF2R* is a large gene (*i.e.*, complementary DNA, 9.1 kb; genomic DNA, >100 kb),^{29,30} only those regions of the receptor known to be involved in ligand binding were screened for mutations. These regions included exons 8 to 10 (repeat 3), exons 27 to 35 (repeats 9-11), and exons 37 to 39 (repeat 13); exon 40 was also assessed for mutations because it was previously shown to contain mutations in other tumors.^{14,19} PCR amplification of DNA extracted from formalin-fixed tissue required multiple primer sets to efficiently span large exons. The exon-specific PCR primers for 2 rounds of seminested PCR amplification of DNA are provided in Table 2. Each round of PCR normally consisted of 35 cycles at 94°C for 15 seconds, 55°C for 5 seconds, and 72°C for 30 seconds, however, annealing temperature varied according to the primers used (Table 2). Direct sequencing of gel-purified PCR products was performed according to the manufacturer's protocol (Thermo Sequence; USB Corporation). Because Taq polymerase infidelity can introduce sequence errors during PCR amplification, all mutations were confirmed by sequenc-

ing the DNA products of 2 or more independent PCR amplifications.

M6P/IGF2R Immunohistochemical Staining. The immunohistochemical technique used to detect M6P/IGF2R in formalin-fixed, paraffin-embedded tissue sections was a modification of our previously described method.³¹ After tissue section deparaffinization, rehydration, and antigen retrieval by microwave treatment (3 × 5 minutes at 750 W in 0.01 mol/L citrate buffer, pH 6.0), the 4-μm sections were incubated for 30 minutes at room temperature with blocking buffer: 2% normal goat serum and 1% milk diluent (Kirkegaard & Perry, Gaithersburg, MD) in buffer (10 mmol/L phosphate-buffered saline, pH 7.4, and 0.1% histochemical grade bovine serum albumin). They were then incubated overnight at 4°C with affinity-purified rabbit polyclonal antibody to bovine M6P/IGF2R (1:1,800 in blocking buffer, provided by Dr. Peter Lobel). A protein concentration of nonimmune rabbit immunoglobulin G (Rockland, Gilbertsville, PA) equal to that of the diluted antibody was used as a control. The tissue sections were then processed according to the recommended procedures provided with the Vectastain rabbit immunoglobulin G Elite ABC kit (Vector Laboratories, Burlingame, CA) using diaminobenzidine tetrahydrochloride as a substrate. The tissue sections were counterstained with hematoxylin after immunohistochemical staining.

Statistical Analysis

Statistical analysis was performed by the χ^2 test; the criterion for statistical significance was the 0.05 level.

Results

M6P/IGF2R Loss of Heterozygosity

Fifty-eight percent (54 of 93) of patients were heterozygous at 1 or more *M6P/IGF2R* polymorphic loci (Fig. 1). A total of 64 HCCs and 4 dysplastic nodules

Table 2. *M6P/IGF2R* Mutation Analysis of Paraffin-Embedded Tissues

Exon*	Amplicon Size (bp)	Tm (°C)	F1 Primer (5' to 3')	R1 Primer (5' to 3')	Nested Primer (5' to 3')†
8-1	139	55	gtggaaaatctgcatgaag	CTCTGTGGCAGGCATACTCAG	R2: GCAGGCATACTCAGTAATCCAC
8-2	158	54	CTAAATCCAAGTCCGCTATG	aggaggcagaaaagcacctg	F2: CAATGCCGCTATGAAATTG
9-1	123	55	gactaagtaagactgtaatctctaatacc	CTGTATTTCAGTTTCTCCACAGAC	F2: aatacctattcatataaaaacaagcctct
9-2	170	50	GATGGAAAAGAATATTTGTTTTATTG	gaaaatcgcacagagggtg	R2: gcacagagggtgttgacg
10	166	55	ttctccaacacacattgtctgt	gcaaaaggaggagggtgaa	R2: tgaactccagcgcacacttac
27-1	120	55	cccttcaactcttccatgttctgt	CCCGGAAGTAATAAGTATTTCGC	R2: ATTCGCCAGCGCTCAGCAT
27-2	112	55	CCTGGGCCTCAACGACAC	GACATGAGGAGACCACCTTGGAC	R2: GACCACCTTGGACTTGTCACTTG
27-3	124	55	GCTTTCCTCAGACGTCTGCC	ttgcaacaaaaggaaaacgacaaaac	R2: acaaaaaggaaaacgacaaaacatggtta
28	83	55	CTCAGAAGCTAACTTATGA	CAGTAGAAGAAGATGGCTGTGGAGC	R2: GGAGCGCTGATAAACCTTATGGC
29	139	46	atgtgcctcttaacttttttag	agttagtctgtccaaaaga	R2: aacacatccatggacttac
30	185	55	acgaccaagcctaactaactg	cacccacagaggaggc	F2: ctgagggtttctctttttcag
31-1	122	54	ctttctgtgtctgtgctgctg	CGTATTTCAGGACAATTATGCCATCT	R2: TTATGCCATCTCCTCAGT
31-2	90	54	GGCAGGGTAAGGGACGGAC	CGGATGGTGGTTGACTTTTTTTC	R2: TTCCGAATCCCATCTGGAC
31-3	112	54	GGCATAATTGCTGAAATACGTTG	gaaggtgcaggaaacagtcct	F2: CCTGAAATACGTGATGGCGAC
32	175	55	caggacctgtctgtcttctgtg	gtgggggttaacgactggcac	R2: aacgactggcaccacctctc
33	165	55	cccatcttctccaccctac	ggacgggagagtcagacaac	R2: acacgagagaccacagcactc
34-1	175	55	cgctttccctgtggtg	GAGATGAGCATGGGCCTA	R2: CTGCTCACGAAATGATCACAC
34-2	169	55	GTCCCTTGCTCCCAATC	tgccatctgaaaactcac	F2: TCCAAATCCGGCTGAGC
35-1	150	48	agcagctttcctaactaac	GGCTGACAAATATTGATG	R2: ATGTAGAAATCAGGGTTG
35-2	159	52	TGAGAGTGAGGATGATGCC	caaaaattatctccacctgt	R2: acccttggattgtcactactac
37-1	122	55	taagaccgtgtcttctctg	CAGGGTACAGCCATCCATCC	F2: cgtgcttctctggcaacag
37-2	121	54	GTCGTCTGCTGATGAAGTG	agcaacgcccagggtgaaac	R2: cccagggtgaaacgacttac
38-1	98	55	actctgtgacggccacgc	CCTCCGTCCTTACAGCCTCCTTG	F2: atggtttttgcccag
38-2	132	55	CCTTTCAGTCCGGGC	CGTAACTAAAACGACCCGCTCATCC	R2: CGACCGCTTATCCTGGTG
38-3	108	50	GCAATCAATGAACTGGATTAC	ctgaagtttattacctcattgc	R2: gtttattacctcattgcaaatatttac
39-1	121	55	cagctgccacactgataatgtt	ACAGCTTCGCCCTGCTC	R2: TTCGCCCTGCTCTCTATGA
39-2	121	55	TCATATTCAATGGGAAGACTACG	gcggagccgctctactc	F2: GGAAGAGCTACGAGGAGTGCA
40-1	164	55	ctctctttccctacactccccag	GTTTCTGGACGAATTTGACTCC	R2: CTCTTTGGAGGGCAGACAAC
40-2	118	55	GGGTGTGATGTGACATTTGAGTGG	gcgggtggactgggaaaggc	F2: GGAGTGCAAATTCGTCAGAAACAC

*Exon number based on Killian et al.³⁰

†Nested primers F2 and R2 should be paired with R1 and F1, respectively.

from informative patients were analyzed for allelic loss. The other liver lesions could not be analyzed for *M6P/IGF2R* loss of heterozygosity because they either resided in noninformative patients or the DNA could not be PCR amplified. Because *M6P/IGF2R* loss of heterozygosity frequency in HCCs was found to be independent of whether the patients lived in southern, central, or northern Japan (data not shown), the data from these 3 geographic regions were combined. Sixty-seven percent (43 of 64) of HCCs and 75% (3 of 4) of dysplastic nodules had *M6P/IGF2R* loss of heterozygosity; there was no significant difference in the frequency between HCCs and dysplastic nodules ($P > .1$). Loss of heterozygosity frequency was also found to be independent of the SNP used for its estimation ($P = .3$) (data not shown), indicating that usually the complete gene is deleted. As previously reported in American liver cancer patients,¹⁶ *M6P/IGF2R* allelic loss was also found in 40% (2 of 5) of phenotypically normal hepatocyte tissue microdissected from the liver adjacent to HCCs with *M6P/IGF2R* loss of heterozygosity. There were no statistically significant associations between the frequency of *M6P/IGF2R* loss of heterozygosity

and the clinicopathologic parameters of patient sex ($P = .8$), presence of cirrhosis ($P = .4$), tumor grade ($P = .5$), and tumor size ($P = .7$) (Table 3). This is consistent with our previous findings that *M6P/IGF2R* loss of heterozygosity is an early event in liver carcinogenesis, and the frequency of allelic loss in HCCs is independent of cirrhosis.^{14,16,26} The role of disease etiology could not be assessed because the majority (*i.e.*, 85%) of the patients were infected with HCV.

M6P/IGF2R Mutations

Missense point mutations and single-base deletions in the *M6P/IGF2R* ligand-binding domains were found in 21% (9 of 43) of HCCs with loss of heterozygosity (Table 4, Figs. 2 and 3). In contrast, none of the dysplastic nodules with *M6P/IGF2R* allelic loss contained comparable second mutations. The frequency of *M6P/IGF2R* mutations is not significantly different ($P = .2$) between dysplastic nodules (0 of 3, 0%), well-differentiated HCCs (4 of 29, 14%), and moderately to poorly differentiated HCCs (5 of 14, 36%), but a trend of increasing *M6P/IGF2R* mutational fre-

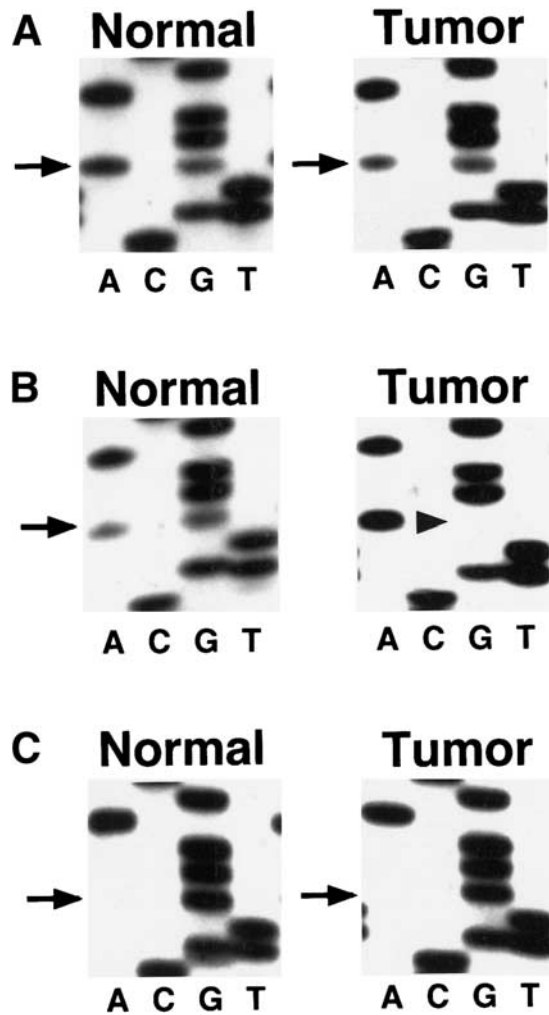


Fig. 1. *M6P/IGF2R* loss of heterozygosity in human hepatocellular carcinomas. A single nucleotide polymorphism (c.1737A→G transition) in exon 12 of the *M6P/IGF2R* (arrow) was used to determine loss of heterozygosity in these representative HCCs. (A) Informative HCC without *M6P/IGF2R* loss of heterozygosity. (B) Informative HCC with *M6P/IGF2R* loss of heterozygosity (arrowhead). (C) Noninformative HCC.

Table 4. *M6P/IGF2R* Loss of Heterozygosity Versus Clinical and Pathological Characteristics

Clinical/Pathological Characteristics	LOH/Informative	Percent LOH
Sex		
Male	35/51	69
Female	11/17	65
Disease Etiology		
HCV	40/58	69
HBV	2/5	40
AIH	1/1	100
Alcohol	1/1	100
HCV + AIH	0/1	0
NBNC	2/2	100
Liver Histology		
Chronic hepatitis	12/20	60
Cirrhosis	34/47	72
Nonspecific reaction	0/1	0
Tumor Grade		
DN	3/4	75
Well HCC	29/40	72
Mod, poor HCC	14/24	58
Tumor size (cm)		
<1.1	11/14	78
1.1-2.0	25/36	69
>2.1	8/12	66
Unknown	2/6	33
Total	46/68	68

Abbreviations: AIH, autoimmune hepatitis; DN, dysplastic nodule; LOH, loss of heterozygosity; Mod, moderately differentiated; NBNC, non-HCV and non-HBV; Poor, poorly differentiated; Well, well differentiated.

quency was observed with tumor grade. *M6P/IGF2R* mutation frequency in HCCs was 43% (3 of 7) for patients in northern Japan (*i.e.*, Iwate, Japan), 20% (1 of 5) for patients in central Japan, and 16% (5 of 31) for patients in southern Japan (*i.e.*, Fukuoka, Japan). These mutation frequencies did not vary significantly ($P = .3$) with the geographical location of the patients in Japan. Nevertheless, single G deletions in the poly G region of *M6P/IGF2R* were only found in HCCs from patients that resided in northern Japan (Table 3, Fig. 3).

Table 3. *M6P/IGF2R* Mutations in Patients With Allelic Loss

Case #	Sex	Age (yr)	Disease Etiology	Tumor Histology (Grade)	Tumor Size (cm)	Liver Histology	<i>M6P/IGF2R</i> Mutation	Sample Source in Japan
1	M	47	HCV	HCC (W)	1.5	Cirrhosis	Exon 27 (3949)*: GAC (Asp) to AAC (Asn)	Fukuoka
2	M	67	HCV	HCC (M)	1.1	Cirrhosis	Exon 34 (5020): ATG (Met) to TGT (Leu)	Fukuoka
3	F	69	HCV	HCC (M)	1.9	Cirrhosis	Exon 31 (4477): CCT (Pro) to ICT (Ser)	Fukuoka
4	M	58	HCV	HCC (W)	1.0	Cirrhosis	Exon 34 (5030): TCC (Ser) to TTC (Phe)	Fukuoka
5	M	66	HCV	HCC (W)	1.3	CH	Exon 28 (4091): GGG (Gly) to GAG (Gln)	Fukuoka
6	M	50	ALD	HCC (M)	5.0	Cirrhosis	Exon 38 (5781): CAC (His) to CAA (Gln)	Toyama
7	M	83	HCV	HCC (M)	6.1	Cirrhosis	Exon 35 (5096): ACC (Thr) to ATC (Ile)	Iwate
8	M	70	HCV	HCC (W)	2.0	Cirrhosis	Exon 28 (4089 to 4096) G deletion	Iwate
9	M	64	HCV	HCC (M)	1.0	Cirrhosis	Exon 28 (4089 to 4096) G deletion	Iwate

Abbreviations: ALD, alcoholic liver damage; CH, chronic hepatitis; M, moderately differentiated; W, well differentiated.

*Nucleotide number based on Morgan et al.²⁹

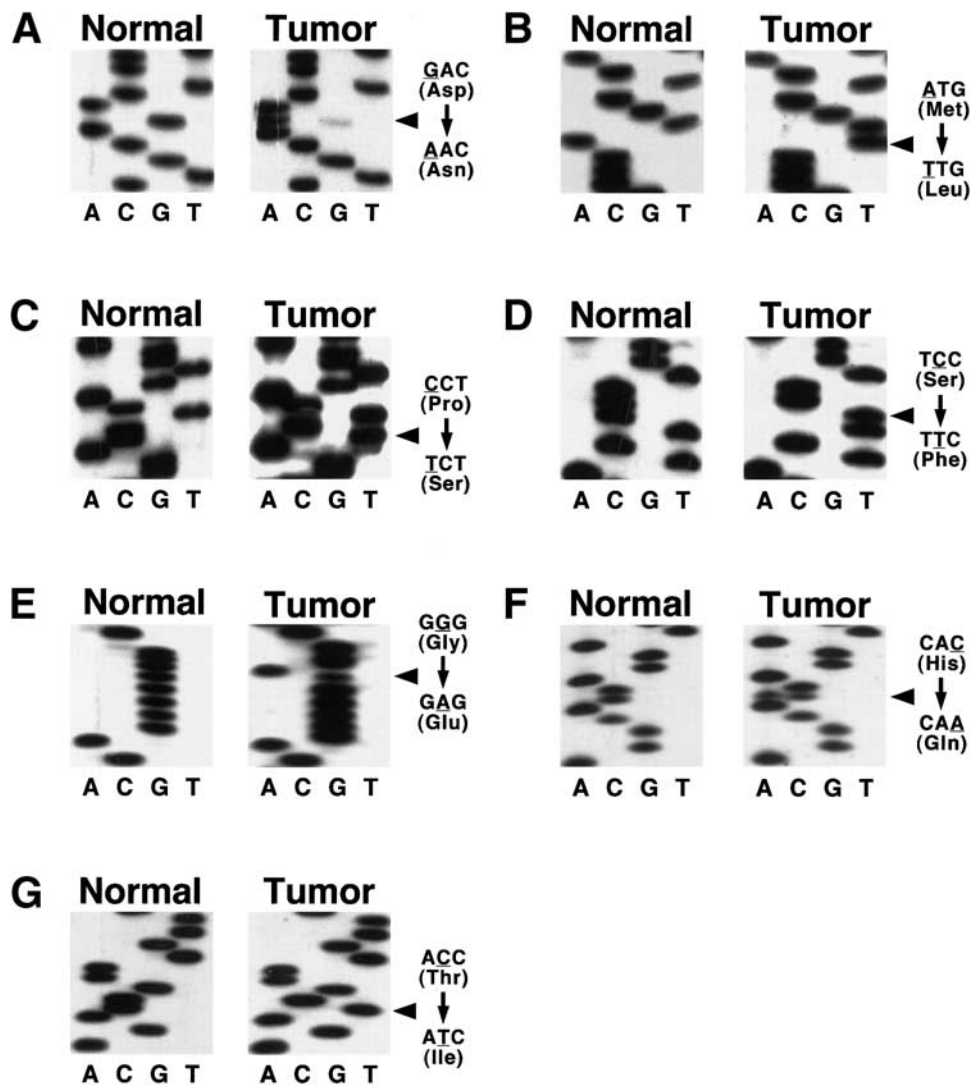


Fig. 2. *M6P/IGF2R* missense point mutations in Japanese hepatocellular carcinomas with loss of heterozygosity. (A) Case 1: c.3949G→A transition, 1268Asp→Asn, exon 27. (B) Case 2: c.5020A→T transversion, 1625Met→Leu, exon 34. (C) Case 3: c.4477C→T transition, 1444Pro→Ser, exon 31. (D) Case 4: c.5030C→T transition, 1628Ser→Phe, exon 34. (E) Case 5: c.4091G→A transition, 1315Gly→Glu, exon 28. (F) Case 6: c.5781C→A transversion, 1878 His→Gln, exon 38. (G) Case 7: c.5096C→T transition, 1650Thr→Ile, exon 35. Normal stromal tissue (normal); hepatocellular carcinoma (tumor). The **arrowheads** show position of point mutations in tumors. DNA sequence has 2 bands at the position of the point mutation in tumor samples A, E, and F because of the presence of contaminating normal tissue. Nucleotide position is based on Morgan et al.²⁹

M6P/IGF2R Immunohistochemical Staining

M6P/IGF2R immunohistochemical staining intensity of 51 HCCs relative to that in the adjacent liver tissue is shown in Table 5; 17 of 68 informative tumors were excluded from analysis because of methodologic difficulties with the immunohistochemical staining. *M6P/IGF2R* staining of HCCs was scored as being either greater than or equal to that in the normal liver (Fig. 4A) or less than that in the normal liver (Fig. 4B). The frequency of HCCs without *M6P/IGF2R* allelic loss (*i.e.*, no loss of heterozygosity) that stained less than normal liver was not significantly different ($P = .3$) than HCCs with allelic loss but no evidence of mutation in the remaining allele (*i.e.*, loss of heterozygosity, no mutation). In contrast, the frequency of HCCs without *M6P/IGF2R* allelic loss (*i.e.*, no loss of heterozygosity) that stained less than normal liver was significantly less ($P = .004$) than HCCs with both alleles mutated (*i.e.*, loss of heterozygosity, mutation).

Therefore, the majority of HCCs with both alleles of the *M6P/IGF2R* mutated have significantly reduced *M6P/IGF2R* staining.

Discussion

We showed in this investigation that *M6P/IGF2R* allelic loss occurs in 68% of HCCs developing in Japanese patients. This frequency of allelic loss is independent of the geographical location of patient residence in Japan and is consistent with that previously reported for HCCs in patients from the United States (61%).¹⁶ Therefore, our findings contrast strikingly with those of Wada et al.²⁴ who found that the *M6P/IGF2R* is not mutated in Japanese HCCs.

One possible reason for this discrepancy is that Wada et al.²⁴ did not microdissect the tumor cells from the tumor tissue before the assessment of *M6P/IGF2R* allelic loss. The inclusion of normal stromal cells in the tumor

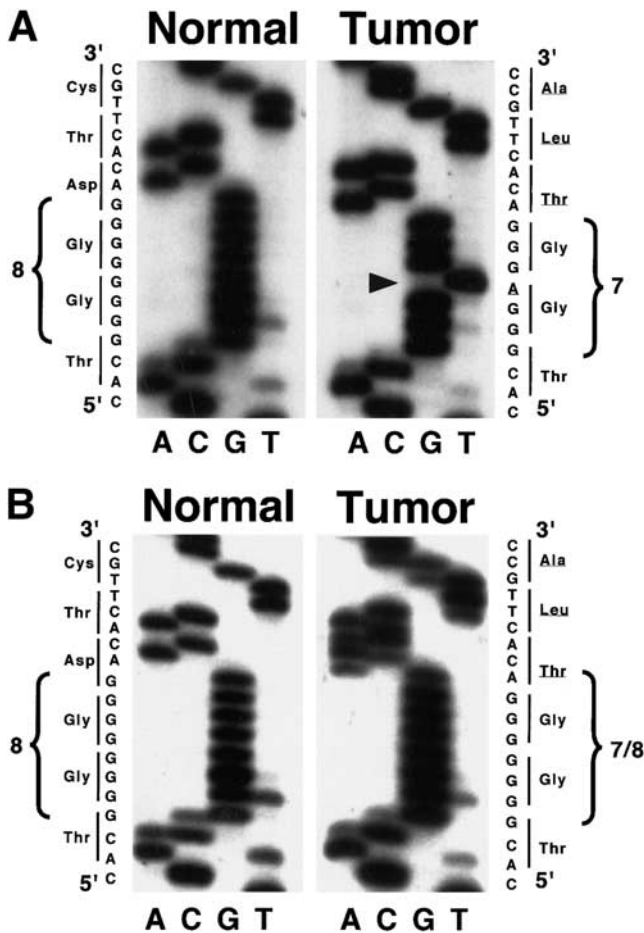


Fig. 3. *M6P/IGF2R* deoxyguanine deletion in a poly(G) repeat sequence in hepatocellular carcinomas with loss of heterozygosity. (A) Case 8: a single G deletion in exon 28. There are 8 deoxyguanines in the repeat region of normal stromal tissue (normal) and 7 deoxyguanines in the repeat region of tumor tissue (tumor). The **arrowhead** shows a c.4092G→T transversion in the polyG repeat region that does not result in an amino acid change. (B) Case 9: a single G deletion in exon 28. There are 8 deoxyguanines in the repeat region of normal stromal tissue (normal) and 7 deoxyguanines in the repeat region of tumor tissue (tumor). DNA sequence for the tumor has double bands from the 3' G until the end of the sequence because normal tissue with an 8 G sequence is contaminating the tumor tissue in which a single G has been deleted from the sequence. Amino acids changed in tumor because of G-deletion are underlined.

sample can easily mask genetic loss of heterozygosity, as previously stressed.³ To circumvent this technical problem, we only screened for *M6P/IGF2R* allelic loss and mutations in microdissected tumor cells. Additionally, the tetranucleotide insertion/deletion polymorphism in the 3'-untranslated region of the *M6P/IGF2R*³² used for loss of heterozygosity analyses is only 27 base pairs away from a GT dinucleotide repeat polymorphism.³³ The tetranucleotide insertion/deletion polymorphism is therefore prone to DNA polymerase slippage artifacts during PCR amplification, thereby reducing the accuracy of allelic loss determination. Consequently, we used 10 re-

cently identified single nucleotide polymorphisms for loss of heterozygosity determination.²⁸

HCCs with *M6P/IGF2R* loss of heterozygosity were also screened for mutations in the mannose 6-phosphate and IGF2 receptor-binding domains of the retained allele; missense mutations and single-base deletions were found in 21% of these tumors. As previously observed in lung cancer, *M6P/IGF2R* mutations were not found in exons 8 to 11. This indicates that the mannose 6-phosphate binding site in repeat 3 is not commonly mutated in HCCs.¹⁰ In contrast, 44% of the observed mutations were in the mannose 6-phosphate binding site in repeat 9 (*i.e.*, exons 27 to 29).¹⁰

Two of these mutations were missense mutations. A c.3949G→A transition that results in an Asn for Asp1268 substitution was identified in exon 27 of an HCC from a patient with HCV-induced cirrhosis. The predicted secondary structure of repeat 9 from the 9 stranded flattened β barrel structure of the cation-dependent mannose 6-phosphate receptor indicates that Asp1268 resides in a conserved domain in the loop region between β strands 2 and 3.^{34,35} A c.4091G→A transition that results in a Glu for Gly1315 substitution was also identified in exon 28 of an HCC from a patient with chronic HCV-induced hepatitis. The substitution of Glu1315, a negatively charged amino acid, for a small uncharged polar amino acid in the putative loop region between β strands 6 and 7³⁴ would be expected to significantly alter the receptor tertiary structure and function. The importance of this amino acid for receptor function is further supported by the finding that Gly1315 has been conserved throughout mammalian evolution.³⁵

The remaining 2 mutations identified in repeat 9 were single-base deletions. The poly deoxyguanosine (poly[G]) region of exon 28 in the *M6P/IGF2R* is a target of microsatellite instability in replication/repair error-positive tumors.¹⁹ We previously detected frameshift mutations in this region in 27% of HCCs from United States patients.¹⁶ In contrast, several studies have reported an absence of mutations in this region of the *M6P/IGF2R* in

Table 5. M6P/IGF2R Immunohistochemical Staining of Japanese HCCs

<i>M6P/IGF2R</i> Mutation	Tumor < Normal*	Tumor \geq Normal†
No LOH	1	15
LOH, no mutation	7	21
LOH, mutation	5	2

NOTE. $P = .004$ by χ^2 test.

**M6P/IGF2R* immunohistochemical staining is less in tumor cells than normal hepatocytes.

†*M6P/IGF2R* immunohistochemical staining in tumor cells is greater than or equal to that in normal hepatocytes.

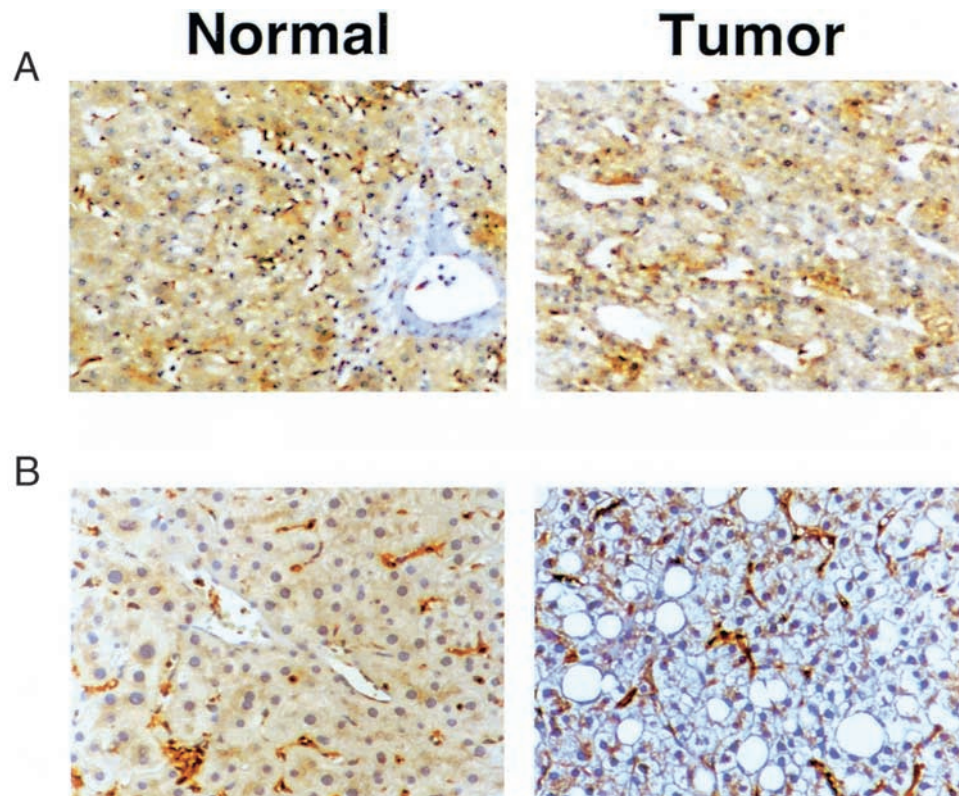


Fig. 4. M6P/IGF2R immunohistochemical staining in hepatocellular carcinomas and adjacent normal liver tissue. (A) M6P/IGF2R expression is positive in a hepatocellular carcinoma with *M6P/IGF2R* loss of heterozygosity and no defined mutation in the remaining allele. (B) M6P/IGF2R expression is negative in a tumor with *M6P/IGF2R* loss of heterozygosity and a c.5030C→T transition in exon 34 of the remaining allele (case 4). Hepatocellular carcinoma (tumor); adjacent normal liver (normal).

HCCs from Japanese patients.^{24,36-38} We found 2 HCCs with single G deletions in the exon 28 poly(G) region, but these frameshift mutations were detected only in patients from northern Japan.

Interestingly, 11 cases of frameshift mutations in the polyG region of the *M6P/IGF2R* have also been reported in gastric, colorectal, and endometrial cancers in the Japanese population.^{18,39-41} The majority of these tumors were also obtained from the northern part of Japan (*i.e.*, Sendai, Iwate, and Sapporo, Japan).^{18,39-41} Historically, there were 2 migrant groups of people that settled in Japan, the Jomonese and the Yayoi.^{42,43} Genetic intermixture between these 2 human populations has occurred since their initial arrival in Japan. Nevertheless, a dual genetic background is maintained even today between the people in northern and southern Japan. Further studies are required to determine if the apparent susceptibility of the polyG region of *M6P/IGF2R* to mutation in tumors results from differences in the genetics of the Japanese who live in northern and southern Japan and/or environmental factors.

Repeat 10 (*i.e.*, exons 30 to 32) of the *M6P/IGF2R* resides between the mannose 6-phosphate and the IGF2 binding domains of the receptor. A Ser for Pro1444 substitution (c.4477C→T transition in exon 31) was identified in Japanese HCCs that is only 4 amino acids from a Gly1449Val mutation frequently observed in HCCs

from the United States.^{14,16,44} Pro1444 is conserved not only in the 3 major mammalian groups (*i.e.*, monotremes, marsupials, and eutherians) but also in avians, strongly indicating that it is important for receptor function.

High affinity IGF2 binding of the M6P/IGF2R requires the presence of both the IGF2 binding site in repeat 11 (*i.e.*, exons 33 to 35) and the enhancer site in repeat 13 (*i.e.*, exons 37 to 39).^{10,45} Two missense mutations were found in repeat 11 of the *M6P/IGF2R*. A 5020A→T transversion that results in a Leu for Met1625 substitution and a c.5096C→T transition that results in an Ile for Thr1650 substitution were identified in exon 35 of HCCs from patients with chronic HCV-induced cirrhosis. Furthermore, a c.5781C→A transversion that results in a Gln for 1878His substitution in repeat 13 was found in an HCC from a patient with alcohol-induced cirrhosis. All 3 amino acids are highly conserved evolutionarily indicating their importance for receptor function.

The mutation frequency for the phosphomannosyl and IGF2 ligand-binding domains of the *M6P/IGF2R* in Japanese HCCs is lower than the 55% mutation frequency detected in HCCs from patients in the United States.¹⁶ The spectrum of missense mutations is also markedly different from those previously reported in both liver and lung cancers from patients in the United States.^{14,16,17} A c.4493G→T transversion in the *M6P/IGF2R* gene that results in a Gly1449Val substitution is

present in approximately 13% of HCCs in the United States, establishing it as a HCC mutational "hot spot."^{14,16} In contrast, this mutation was absent in all the analyzed HCCs from Japanese patients. Approximately 80% of *M6P/IGF2R* missense mutations detected in liver and lung tumors in United States patients occur in glycines located within predicted transition loop regions between putative β strands of the receptor^{14,16,17,46}; however, only 10% of mutations in Japanese HCCs involved glycine. These mutational differences between HCCs in patients from the United States and Japan might be because of differences in size or grade of HCCs, the different genetic backgrounds of the 2 human populations, or environmental factors.

We also immunohistochemically stained HCCs and adjacent liver tissues with a M6P/IGF2R-specific antibody to assess receptor levels. M6P/IGF2R was detected in most HCCs; however, the level in the HCC versus that in the adjacent liver tissue was tumor dependent. As expected, M6P/IGF2R expression in HCCs was greater than or equal to that in the adjacent liver in the majority of tumors without *M6P/IGF2R* loss of heterozygosity. M6P/IGF2R expression in HCCs was also confirmed to be significantly less than that in the adjacent liver in most HCCs with both alleles mutated. However, approximately 25% of HCCs with *M6P/IGF2R* loss of heterozygosity and no ligand binding site mutations in remaining alleles also had lower receptor expression in the tumor than in the adjacent liver. This indicates that approximately 1 quarter of Japanese HCCs have undetected structure altering mutations residing in the 70% portion of the *M6P/IGF2R* that was not sequenced in this study. Interestingly, this still leaves about half of HCCs with *M6P/IGF2R* loss of heterozygosity with apparently only 1 allele inactivated.

One reason for only a single *M6P/IGF2R* allele being inactivated in some HCCs is that there may be other presently unknown tumor suppressor genes located on 6q that are also mechanistically involved in HCC formation. Alternatively, *M6P/IGF2R* haploid insufficiency may itself provide hepatocytes chronically infected with HBV or HCV with a selective proliferative and/or survival advantage, thereby resulting in the formation of clonal lesions of preneoplastic cells from which HCCs ultimately develop.¹⁶ This is consistent with haploid insufficiency of other tumor suppressor genes, such as *Nf2*, *p27^{Kip1}*, *p53*, *Ptch*, *Pten*, and *TGF- β* , promoting tumor formation (reviewed in Islam and Islam⁴⁷).

M6P/IGF2R is normally imprinted in mice with only the maternal copy of the gene being expressed.⁴⁸ In contrast, both copies of the *M6P/IGF2R* are expressed in humans because genomic imprinting at this locus was lost

in a common ancestor to the primate lineage of mammals approximately 75 million years ago.⁴⁹ Importantly, restoration of biallelic *M6P/IGF2R* expression in mice results in a marked reduction in offspring weight late in embryonic development that persists into adulthood.⁵⁰ These findings show that *M6P/IGF2R* allelic loss or haploid insufficiency significantly enhances cell growth during fetal development. This predicts that the loss of even a single *M6P/IGF2R* allele may also promote cell growth in human somatic cells. This intriguing postulate can now be experimentally tested by comparing liver cancer susceptibility of wild-type mice, which are functionally haploid at the *M6P/IGF2R* locus because of imprinting,⁴⁸ with that of mice with 2 functional *M6P/IGF2R* alleles.⁵⁰

In conclusion, *M6P/IGF2R* mutation occurs in Japanese HCCs with a frequency comparable with that found in the United States, establishing its worldwide prominence as a liver tumor-suppressor gene. The similar frequency of *M6P/IGF2R* allelic loss in HCCs and dysplastic nodules in Japanese patients provides further evidence that haploid insufficiency at this locus is an early event in human liver carcinogenesis.

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