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RAPID COMMUNICATION

Clinical significance of loss of heterozygosity for M6P/IGF2R in patients with primary hepatocellular carcinoma

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Abstract

AIM: To investigate the relationship between loss of heterozygosity (LOH) for mannose 6-phosphate/insulin-like growth factor 2 receptor (M6P/IGF2R) and the outcomes for primary HCC patients treated with partial hepatectomy.

METHODS: The LOH for M6P/IGF2R in primary HCC patients was assessed using six different gene-specific nucleotide polymorphisms. The patients studied were enrolled to undergo partial hepatectomy.

RESULTS: M6P/IGF2R was found to be polymorphic in 73.3% (22/30) of the patients, and of these patients, 50.0% (11/22) had tumors showing LOH in M6P/IGF2R. Loss of heterozygosity in M6P/IGF2R was associated with significant reductions in the two year overall survival rate (24.9% *vs* 65.5%; $P = 0.04$) and the disease-free survival rate (17.8% *vs* 59.3%; $P = 0.03$).

CONCLUSION: These results show M6P/IGF2R LOH predicts poor clinical outcomes in surgically resected primary HCC patients.

Key words: Loss of heterozygosity; Mannose 6-phosphate/insulin-like growth factor 2 receptor; Hepatocellular carcinoma

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common type of malignancy, being especially prevalent in the Southeast Asian and sub-Saharan African populations[1]. Etiological risk factors for HCC formation include hepatitis virus infection, alcohol consumption and dietary exposure to aflatoxin B1[1,2]. In Korea and Taiwan, approximately 90% of all patients with HCC are Hepatitis B surface antigen (HbsAg)-positive, and prospective studies have found Hepatitis B virus carriers have a 200-fold increase in relative risk for HCC. The mannose 6-phosphate/insulin-like growth factor 2 receptor (M6P/IGF2R) is mapped at the chromosome location 6q25-27[3], which is predicted to contain a liver tumor suppressor gene[4]. This gene encodes a receptor which functions in intracellular lysosomal enzyme trafficking, transforming growth factor beta (TGF-β) activation, and IGF2 degradation[5]. 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"[6]. Elevated IGF2 levels during murine development arising from M6P/IGF2R deficiency result in cardiac abnormalities, cleft palate, fetal overgrowth and prenatal lethality[7]. Furthermore, the large-offspring syndrome found in cloned animals is frequently associated with epigenetic changes in M6P/IGF2R imprinting regulation which result in decreased gene expression[8]. Thus, M6P/IGF2R plays a crucial role in regulating mammalian fetal growth and development. M6P/IGF2R is also mechanistically involved in the genesis of human cancer[9-12]. M6P/IGF2R loss of heterozygosity (LOH), coupled with intragenic loss-of-function mutations in the remaining allele, is a common event in human cancers[6,10]. Tumor cell growth is inhibited when M6P/IGF2R expression is restored to normal, whereas it is increased when gene expression is reduced[13-16]. The results of

these mutational and functional studies clearly show M6P/IGF2R possesses the characteristics necessary to be classified as a tumor suppressor gene[17]. Our results show M6P/IGF2R LOH in primary HCC patients predicts poor therapeutic outcomes.

MATERIALS AND METHODS

Patients

Paraffin-embedded tissue sections from 30 patients, who were confirmed histopathologically to have HCC, were obtained from the Gyeongsang National University and the Catholic University of Korea. All patients had a history of hepatitis virus infection and/or cirrhosis, and had undergone partial hepatectomy for the treatment of their disease.

Tissue microdissection and loss of heterozygosity analysis for M6P/IGF2R

Microdissection of 10-μm histology sections from tumor tissue and the surrounding normal liver tissue was performed as described in previous studies[18,19]. Briefly, paraffin-embedded sections were deparaffinized in xylene (2 × 5 min), exposed for 2 min to graded ethanol washes (namely, 100%, 95%, 70% and 50% ethanol) and rehydrated in H_2O before staining. The tissue sections were then stained for 30 s with 2% methylene blue and rinsed in H_2O before allowing them to air dry. Tumor tissue and the surrounding normal tissue (50-cells) were carefully microdissected using a serial section stained with hematoxylin-eosin for comparison. The normal tissue used for genotyping was connective tissue. The dissected tissues were then placed in 75 μL of Tris-ethylenediamine-tetraacetic acid buffer (10 mmol/L Tris-HCl, pH 8.0 at 25℃ and 0.5 mmol/L ethylenediamine tetraacetic acid, pH 8.0 at 25℃ containing 5 μL of 20 g/L proteinase K (Boehringer Mannheim, Indianapolis, IN). This mixture was incubated at 52℃ for 3 h and then at 85℃ for 10 min. Polymerase chain reaction (PCR) analysis was conducted using 5 μL of this mixture, as described below. Six single nucleotide polymorphisms (Table 1), identified as c. 901C > G (exon 6), c. 1197A > G (exon 9), c. 1737G > A (exon 12), c. 2286A > G (exon 16), c. 6206A > G (exon 40) and c. X47-5t > a (exon 47), were also analyzed following 2 rounds of nested PCR. The exon-specific forward and reverse primers have been previously described[19,20]. The exons containing these polymorphisms were amplified by PCR from genomic DNA under conditions identical to those described above. The single nucleotide polymorphisms used to determine the loss of heterozygosity in M6P/IGF2R were assessed by directly sequencing the PCR products according to the manufacturer's protocol (Thermo Sequenase, USB Corporation, Cleveland, OH). Taq DNA polymerases may introduce sequence errors during PCR amplification, and unequal amplification of the two alleles can result in the false positive detection of a loss of heterozygosity. Thus, both normal and tumor DNA templates were amplified in three independent PCR reactions, and assessed for LOH in M6P/IGF2R.

Due to the potential for contaminating the tumor tissue sample with normal stroma, allele loss in informative patients was defined as a > 50% decrease in the ratio of the two alleles in tumor tissue *versus* that in the surrounding normal stromal tissue. This was quantified using a densitometer.

Statistical analysis

Overall survival and disease-free survival rate represented the clinical end-point. All curves were computed using the Kaplan-Meier method starting from the time of study entry. Curves for different sub-groups were compared by the Cox-Mantel test. A chi-squared test was used to compare the clinical characteristics between M6P/IGF2R-informative and M6P/IGF2R-excluded patients, and between informative patients with and without LOH in M6P/IGF2R. A $P < 0.05$ was considered to be statistically significant.

RESULTS

Analysis of LOH in M6P/IGF2R

The study population consisted of a total of 30 patients who were enrolled in a retrospective clinical trial for primary HCC from March 1999 to June 2003 (Table 2). Among the 30 patients, 22 (73.3%) were informative (that is, polymorphic), and the tumors in 50% (11/22) of these patients exhibited LOH in M6P/IGF2R (Figure 1). There was no significant difference between M6P/IGF2R-informative patients and those not used in this study in terms of the clinical characteristics of sex, age, liver cirrhosis, tumor of differentiation, size of tumor, or type of hepatitis.

Clinical outcome

The median follow-up for surviving patients enrolled in this trial was 33 mo (range: 2 to 62 mo). The median survival times were 34 mo and 23 mo for overall survival and disease free survival, respectively. There was no relationship between LOH in M6P/IGF2R and clinical factors, such as sex, age, liver cirrhosis, tumor differentiation, tumor size, or the type of hepatitis. The median overall survival times in patients with and without LOH in M6P/IGF2R were 18 mo and 44 mo, respectively, and the two year overall survival rates were 24.9% and 65.5%, respectively (log-rank, $P = 0.04$) (Figure 2A). Likewise, the median disease-free survival rates in patients with and without LOH in M6P/IGF2R were 12 mo and 36 mo, respectively, and the 3 year disease-free survival rates were 17.8% and 59.3%, respectively (log-rank, $P = 0.03$) (Figure 2B). The clinical relevance of LOH in M6P/IGF2R to both overall survival and disease-free survival rates was confirmed in the analysis ($P < 0.05$ for both comparisons). These results indicate that LOH in M6P/IGF2R results in poor patient outcome when surgical resection is employed, since all other measured clinical characteristics of the primary HCC patients were comparable to those in patients with a non-mutated M6P/IGF2R tumor suppressor gene.

DISCUSSION

M6P/IGF2R LOH occurs frequently in human breast, liver and lung cancer[9,10,20]. Mutation in M6P/IGF2R is

Table 1 M6P/IGF2R LOH analysis of paraffin-embedded tissue

Position	Nucleotide	Genotype	Amplicon size (bp)	F1 primer (5'-3')	R1 primer (5'-3')	Nested primer (5'-3')
Exon 6	901	C/G	91	CACCAGGCGTTTGATGTTGG	CTCCAGCAAGGACCTGACTTTC	CCTCCGATGCTGTTGGCGT
Exon 9	1197	A/G	123	ACTAAGTAAGACTGTAATCTTCTAATACC	GTCTGTGGAGAAACTG AAATACAG	AATACCTATTCATATAAAACAAGCCTC
Exon 12	1737	G/A	111	TATTTGTCACAGAGTGCTGCAGG	GGCATCCAGTTTGGAATGAGTTAG	GGAAGATCTAGGTGATGCTTTTC
Exon 16	2286	A/G	187	GAAGCTTTCATATTATGATGGGATG	GAGGATACTCATGCCTGTGGTG	CATCGCGCTCCCTGAGGATACT
Exon 40	6206	A/G	118	GGGTGTGATGTGACATTTGAGTGG	GCCTTCCCAGTCCACCCGC	GGAGTGCAAATTCGTCCAGAAAC
Exon 47	X47-5	t/a	161	ATGCCCTCTCTACACTGGAGTA	GTAAGCTGACCACTTGCTGTAGG	CAGTGATAAGTAAGCTGACC

Table 2 Patient characteristics *n* (%)

Characteristics	Total patients (*n* = 30)	Informative patients (*n* = 22)
Age: yr	36-78 (Median: 58)	39-78 (Median: 59)
Gender: male/female	22 (73.3)/8 (26.7)	16 (72.7)/6 (27.3)
Disease etiology		
HBV	18 (60.0)	15 (68.2)
HCV	6 (20.0)	3 (13.6)
Alcohol	6 (20.0)	4 (18.2)
Tumor Grade		
Well differentiation	9 (30.0)	6 (27.3)
Moderate differentiation	11 (36.7)	8 (36.4)
Poor differentiation	10 (33.3)	8 (36.4)
Liver Histology		
Chronic hepatitis	3 (10.0)	2 (9.1)
Cirrhosis	21 (70.0)	16 (72.7)
Nonspecific reaction	6 (20.0)	4 (18.2)
Tumor size (cm)		
< 2	4 (13.3)	2 (9.1)
2-5	14 (46.7)	11 (50.0)
> 5	12 (40.0)	9 (40.9)
No. of Tumor		
Single	18 (60.0)	12 (54.5)
Multiple	12 (40.0)	10 (45.5)
AJCC Stage		
Ⅱ	18 (60.0)	12 (54.5)
ⅢA	12 (40.0)	10 (45.5)

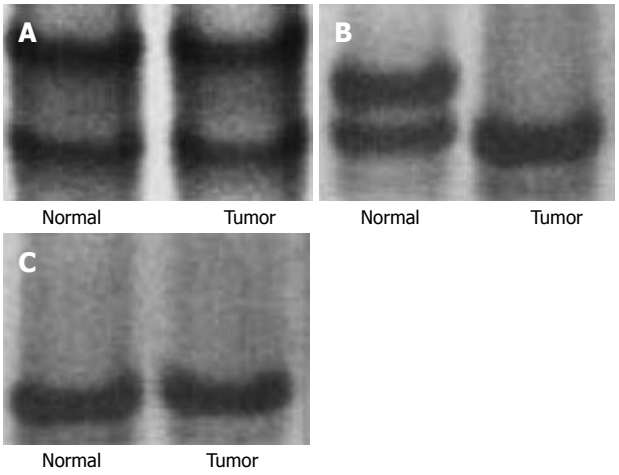

Figure 1 There are three results in the LOH analysis for M6P/IGF2R in primary hepatocellular carcinoma (HCC). **A**: Informative HCC without LOH in M6P/IGF2R; **B**: Informative HCC with LOH in M6P/IGF2R; **C**: Non-informative HCC.

also commonly found in gastrointestinal and gynecological cancers, because the coding sequence of M6P/IGF2R contains a poly-G region, which is a mutational target in tumors with mismatch repair deficiencies and microsatellite instability[12,21]. Functional studies show the introduction of an exogenous wild-type M6P/IGF2R with a single inactivated allele into human colorectal cancer cells significantly decreases cell growth rate and enhances apoptosis[13]. Conversely, the loss of M6P/IGF2R expression promotes cancer cell growth by increasing intracellular signaling from both the receptors, the insulin-like growth factor 1 receptor and the insulin receptors[22]. Kong *et al*[10] demonstrated mutations in both alleles of the M6P/IGF2R are found in more than 50% of squamous cell carcinomas of the lung. In the present study, we demonstrated M6P/IGF2R LOH in primary HCC is also associated with poor patient prognosis. Loss of heterozygosity in malignancy can also occur due to chromosomal deletion or somatic recombination resulting in uniparental disomy[23]. Because chromosomal deletion can affect more than one gene, M6P/IGF2R LOH does not rule out the possibility of a loss of adjacent genes with tumor suppressor functions in HCC. This study also showed improvements in overall survival and disease-free survival in those patients undertaking surgical resection for primary HCC with M6P.IGF2R LOH. It was found patients with mutations in M6P/IGF2R had a significantly worse prognosis than those who had a non-mutated M6P/IGF2R allele.

M6P/IGF2R is normally imprinted in mice, with only the maternal copy of the gene being expressed[24]. By contrast, both copies of M6P/IGF2R are expressed in humans, because genomic imprinting at this locus was lost in the primate lineage approximately 70 million years ago[25]. Importantly, the restoration of biallelic M6P/IGF2R expression in mice results in a marked reduction in offspring weight late in embryonic development that persists into adulthood[26]. This demonstrates that M6P/IGF2R allelic loss or haploid insufficiency markedly enhances cell proliferation and/or survival during fetal development. Therefore, the mutation of even a single allele of M6P/IGF2R in human somatic cells is predicted to promote cell growth. Haploid insufficiency of tumor suppressor genes, such as Nf2, $p27^{Kip1}$, p53 and TGF-β, is known to promote tumor formation[27-29]. Yamada *et al*[9] demonstrated that, in patients chronically infected with Hepatitis B and/or Hepatitis C viruses, mutations

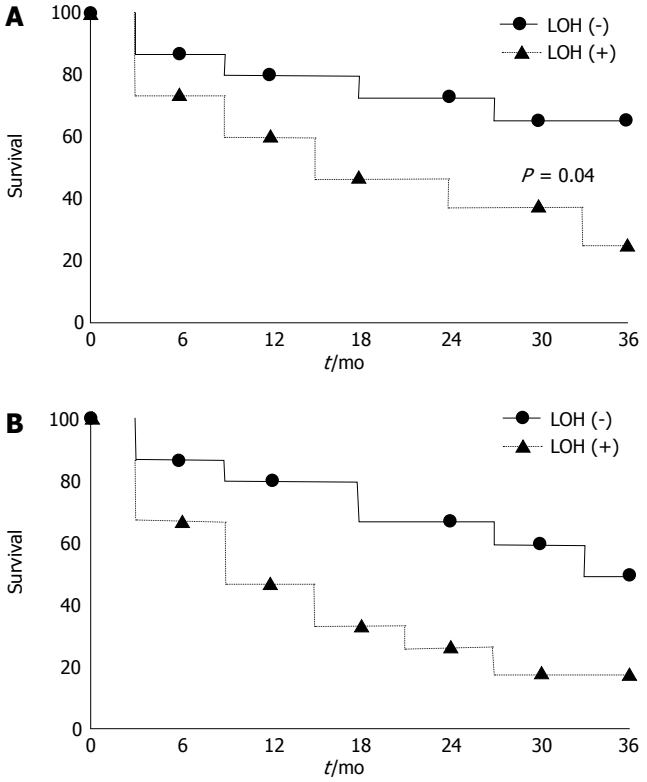

Figure 2 **A:** The overall survival according to loss of heterozygosity in M6P/IGF2R; **B**: The disease free survival according to loss of heterozygosity in M6P/IGF2R.

in M6P/IGF2R take place not only in HCC, but also in the phenotypically normal hepatocytes adjacent to these tumors. Interestingly, only one M6P/IGF2R allele is inactivated in the adjacent cirrhotic tissue, even when both alleles are mutated in the HCC. These findings are consistent with the normal appearing, preneoplastic hepatocytes forming clonal masses in the liver, because M6P/IGF2R haploid insufficiency affords them with a selective growth and/or survival advantage relative to normal hepatocytes[30].

In conclusion, this study shows the analysis of M6P/IGF2R LOH provides clinical significance in surgically resected primary HCC patients.

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COMMENTS

Background

The associated nature of hepatocellular carcinoma (HCC) and mannose 6-phosphate/insulin-like growth factor 2 receptor (M6P/GF2R) is well reported. However, there is scant research on the clinical significance of loss of heterozygosity (LOH) in M6P/GF2R in patients with primary HCC. In the present study, we aimed to investigate the relationship between LOH for M6P/IGF2R and various factors, including survival rate, in primary HCC patients treated with partial hepatectomy.

Research frontiers

Several studies have investigated various types of cancer, including HCC, in which LOH for M6P/IFG2R might appear. We studied the relationship between LOH for M6P/IGF2R and HCC, and confirmed the survival rate is directly related to the LOH for M6P/IGF2R.

Innovations and breakthroughs

The present research studied cases with primary HCC, but research on the usefulness of LOH for M6P/IGF2R should be continued by comparing cases with metastatic HCC, cholangiocarcinoma and other tumors.

Applications

The results of this study suggest the presence of LOH for M6P/IGF2 may represent some poor prognostic factors in primary HCC patients treated with hepatectomy.

Peer review

The paper represents a real advance in the loss of heterozygosity for M6P/IGF2R in patients with primary HCC. The conclusions are valuable. The methodology is correct and the results are well presented.

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