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Imbalance in Expression Levels of Insulin-like Growth Factor 2 and H19 Transcripts Linked to Progression of Hepatocellular Carcinoma

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Abstract. *Background: The imprinted genes insulin-like growth factor 2 (IGF2) and H19 play important roles in various malignancies including hepatocellular carcinoma (HCC). Materials and Methods: We used DNA microarray and patient follow-up data to examine the relationship between expression of IGF2 and H19 and HCC. Results: We found that imbalances in levels of IGF2 and H19 transcripts were correlated with advanced tumor stage and poor outcome in HCC patients. Conclusion: In addition to their known epigenetic or genetic abnormality in malignancies, these findings suggest that altered transcription of these two imprinted genes contributes to progression of HCC.*

Epigenetic changes in the genome are hallmarks of the pathogenesis of many malignancies (1). Recently, epigenetic dysregulation of the 11p15 locus has attracted attention as one of several pathways in hepatocarcinogenesis (2-4). Among a dozen genes localized to 11p15, epigenetic abnormalities in the *insulin-like growth factor 2 (IGF2)* and *H19* genes have been observed in hepatocellular carcinoma (HCC) (4). In most normal adult tissues, only the paternal allele of *IGF2* is expressed, whereas only the maternal allele of *H19*, which is located close to *IGF2*, is expressed. This mechanism of gene regulation is known as genomic imprinting (1, 6-10).

It has been reported that, during hepatocarcinogenesis, increased expression of the *IGF2* gene is associated with loss of adult-type promoter (P1) transcription, re-imprinting of the fetal-type promoters (P2-P4) and expression of both alleles of the *H19* gene (2, 11-16). Biallelic expression of the *IGF2* gene in HCC has also been observed (17). Despite significant efforts in this field, it remains unclear how levels of *IGF2* and *H19* expression are related to progression of HCC because there is little quantitative information for both *IGF2* and *H19* in a larger cohort. To clarify the roles of these genes in the progression of HCC, we used DNA microarray data of HCC samples (18-22).

Materials and Methods

DNA microarray data. We previously performed DNA microarray analyses of 60 HCC samples from 60 patients (18-22). All of them were followed for more than 3 years after curative hepatectomy. Of these 60 patients, one (sample ID: nonBC03T) who died of acute pancreatitis after surgery was excluded from the present study. As a result, the remaining 59 patients were subjected to the present study. The raw data for *IGF2* (GenBank accession number HG3543-HT3739) and *H19* (GenBank accession number M32053) and clinicopathological characteristics are available at URL (http://surgery2.med.yamaguchi-u.ac.jp/research/DNAchip/hcc-recurrence/index.html), which are released according to Minimum Information About a Microarray Experiment (MIAME) proposed by Brazma *et al.* (23).

Microarray data for *IGF2* (18) and *H19* (data not shown) in tumor were validated by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis.

Abbreviations: HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus.

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Key Words: Microarray, hepatocellular carcinoma, IGF2, H19, imprinted gene.

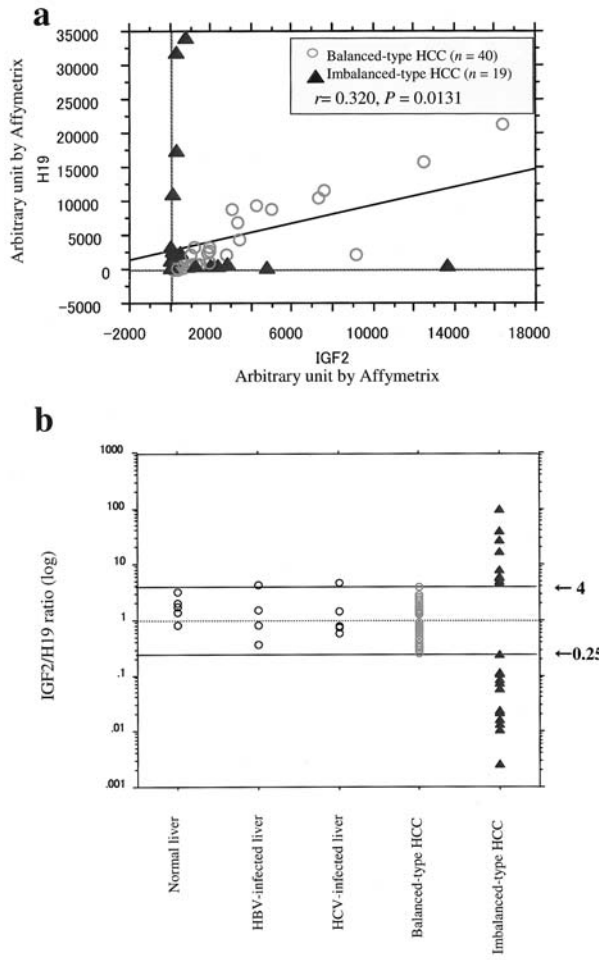

Figure 1. *Expression of IGF2 and H19 in 59 HCCs and 16 control livers. (a), Coordinated expression of IGF2 and H19 in HCCs. IGF2 and H19 genes showed coordinated expression in the 59 HCCs (r=0.32, p=0.0131). However, two subtypes were evident: HCC with coordinately expressed IGF2 and H19 (balanced-type, circle) and HCC with imbalanced expression (imbalanced-type, triangle). (b), Distribution of balanced- and imbalanced-type HCCs based on the IGF2: H19 expression ratio. Sixteen control livers were subjected to DNA microarray analyses and microarray data for IGF2 and H19 were extracted (data not shown). The IGF2: H19 expression ratio in the16 control livers was 2.028±0.38 (mean±SE; range, 0.372 to 4.858). The ratios for all 40 balanced-type HCCs (circles) ranged from 0.25 to 4 and were similar to those for most control livers. In contrast, the ratios for the19 imbalanced-type HCCs (triangles) were less than 0.25 or greater than 4.*

Control livers. Sixteen non-tumorous livers, handled as follows, were used as controls. Six non-tumorous liver samples were obtained from patients who underwent hepatic resection for benign or metastatic liver tumors. All six livers were histologically normal and were seronegative for both hepatitis B surface antigen and hepatitis C virus (HCV) antibody. Five hepatitis B virus (HBV)-infected and five HCV-infected liver samples were obtained from the non-tumorous areas of ten patients with HCC. These 16 non-tumorous livers were also subjected to the DNA microarray analysis (24).

Table I. *Imbalance in expression levels of IGF2 and H19 and progression of HCC.*

Factors	Imbalanced type (n=19)	Balanced type (n=40)	*P* value
Sex			p=0.595*
Male (n=43)	13	30	
Female (n=16)	6	10	
Age (year)	65.2±1.6	61.8±1.4	p=0.143**
Virus type			p=0.764***
B type (n=11)	4	7	
C type (n=40)	12	28	
Non-B, non-C (n=8)	3	5	
Tumor size (cm)	4.8±0.6	3.7±0.4	p=0.092**
Tumor differentiation			p=0.099***
Well (n=5)	3	2	
Moderately (n=44)	11	33	
Poorly (n=10)	5	5	
pTNM Stage			p=0.015***
I (n=22)	7	15	
II (n=27)	5	22	
IIIA (n=10)	7	3	
Venous invasion			p=0.193*
(-) (n=38)	10	28	
(+) (n=21)	9	12	

*Chi-square test
**Student's *t*-test
***Fisher's exact test
Virus type; B type, HBV Ag(+)/HCV Ab(-);C type, HCV Ab(+);non-C; HBV Ag(-)/HCV Ab(-)
pTNM Stage was determined according to the International Union Against Cancer TNM classification (2002).
Imbalanced-type and balanced-type; see legend of Figure 1.

Statistical analysis. The Pearson correlation coefficients (r) were calculated for the expression levels of *IGF2* and *H19* in 59 HCC samples. The Chi-square test, Student's *t*-test, and Fisher's exact test were used to analyze differences in clinicopathological characteristics between HCC with coordinately expressed *IGF2* and *H19* (n=40) and that with imbalanced expression of *IGF2* and *H19* (n=19). All relapse-free survival data were analyzed by the Kaplan-Meier method using the log rank test for statistical significance. $P<0.05$ was considered significant.

Results

Our quantitative data showed that *IGF2* levels were correlated positively with *H19* levels in the 59 HCC samples (r=0.320, p=0.0131, Figure 1a), which was consistent with the results of previous studies (11, 14, 18). We identified two subtypes of HCC: that with coordinately expressed *IGF2* and *H19* (n=40, balanced type, Figure 1a, circle) and that with imbalanced

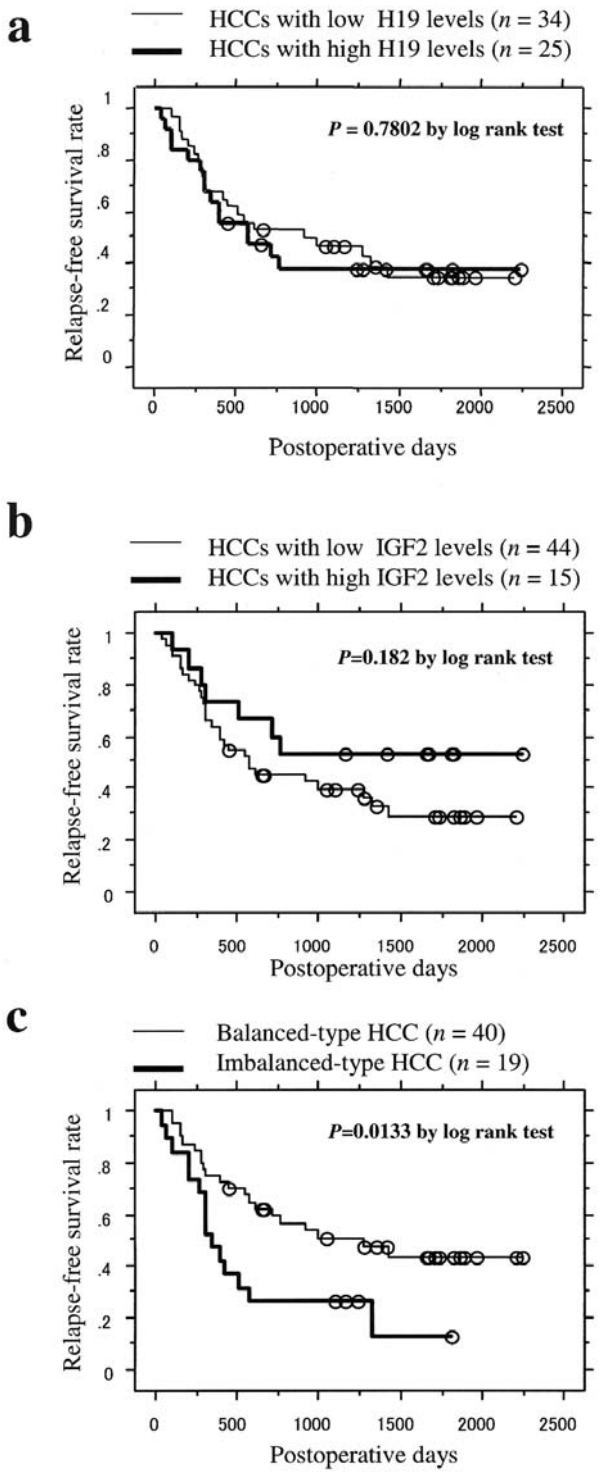

Figure 2. *Relationship between IGF2 and H19 levels and relapse-free survival in 59 HCC patients. (a), IGF2 levels and relapse-free survival. (b), H19 levels and relapse-free survival. (c), Imbalance in expression levels of IGF2 and H19 and relapse-free survival.*
Neither IGF2 nor H19 alone was correlated with relapse-free survival (a, b). In contrast, an imbalance in expression of IGF2 and H19 genes was correlated with relapse-free survival (c).

expression of *IGF2* and *H19* (n=19, imbalanced type, Figure 1a, triangle). To understand better the clinicopathological features of these two subtypes, we compared *IGF2* and *H19* expression data for tumors with those for 16 non-tumorous liver specimens (controls). The *IGF2*/*H19* expression ratio in control livers was 2.028±0.38 (mean±standard error (SE), range, 0.372 to 4.858), and this ratio was higher than 0.25 or lower than 4 in most controls (Figure 1b), suggesting that this distribution represents individual differences in the levels of expression of these genes. The *IGF2*/*H19* expression ratio in all balanced-type HCCs (40 out of 59 HCCs) showed the same pattern of distribution as that in the controls (Figure 1b). In contrast, the distribution of the *IGF2*/*H19* expression ratio in all imbalanced-type HCCs (19 out of 59 HCCs) differed from that of controls (Figure 1b). This result prompted us to evaluate differences in progression-related characteristics between the balanced- and imbalanced-type HCCs (Table I). We found that imbalanced-type HCCs tended to be more advanced than balanced-type HCCs (Table I).

We next investigated the relationship between the expression of *IGF2* and *H19* and relapse-free survival of HCC patients. Levels of expression of *IGF2* and *H19* in 16 control livers were 2253±160 (mean±SE) and 1795±356, respectively (arbitrary unit from Affymetrix). Expression was characterized as "high" when the level in tumor was higher than the mean value for control livers. High *IGF2* expression was observed in 15 (25.4%) out of 59 HCCs. High expression of *H19* was detected in 25 (42.4%) out of 59 HCCs. There was no difference in relapse-free survival between HCC with increased *IGF2* levels and that without (Figure 2a). HCCs with high *H19* expression were at more advanced stages than those without (data not shown); however, there was no difference in relapse-free survival between HCC with increased *H19* levels and that without (Figure 2b). In contrast, an imbalance in expression levels of the *IGF2* and *H19* genes was correlated with short relapse-free survival (Figure 2c).

Discussion

HCC is one of the most common cancers with an estimated 564,000 new cases in 2000 (25), representing a major international health problem because the incidence is increasing in many countries. The fact that the outcome of HCC patient remains poor is due in part to the low resectability rate at the time of the diagnosis (26) and the high frequency of intrahepatic recurrence (19), suggesting the concept of a multimodal approach to treatment (27). Many genes and gene products, such as p53 (28), nm23 (29), telomerase (30) and vascular endothelial growth factor (31), have been identified as being associated with the metastatic potential of HCC. These allow us to better understand the molecular mechanisms of the recurrence and will enable us to develop novel therapeutic options for improving poor

prognoses. In this regard, our present study adds altered levels of imprinting genes *H19* and *IGF2* to the mechanism underlying HCC metastasis that had been found previously.

Many studies have suggested that epigenetic and genetic changes at the *IGF2* and *H19* loci are involved in hepatocarcinogenesis (2, 11-17). Sohda *et al.* (14) reported that these genes were coordinately overexpressed in 37% of HCCs compared with non-tumorous livers. Li *et al.* (11) reported that *H19* and *IGF2* in HCC were regulated in parallel but that their expression levels were variable. Tannapfel *et al.* (32) performed protein microarray analyses and found increased levels of IGF2 in HCC in comparison with non-tumorous liver. However, the possible relationship of elevated IGF2 protein to progression of HCC was not examined (32). Thus, to our knowledge, there have been no studies of the relationship between *IGF2* and *H19* transcript levels and HCC progression.

Our present data indicate that imbalanced-type HCCs are more advanced than balanced-type HCCs and that the relapse-free survival rate is significantly lower in imbalanced-type HCCs in comparison to balanced-type HCCs. However, neither *IGF2* nor *H19* alone was correlated with relapse-free survival of HCC patients. Our present results suggest that these genes have little or no functional contribution to the progression of HCC. Rather, it is possible that changes in transcriptional regulation of *IGF2* and *H19* are related to the progression and metastatic potential of HCC. DNA methyltransferase (DNMT) 1 is a major enzyme involved in establishing genomic methylation patterns. It was shown that the relapse-free survival rates of patients with HCCs exhibiting increased DNMT1 protein expression were significantly lower than those of patients with HCCs that did not exhibit increased expression (33). Thus, the metastatic potential of HCC cells may be largely attributable to the epigenetic condition of many genes including *IGF2* and *H19*.

The roles of *IGF2* and *H19* in neoplastic transformation are enhanced by specific factors produced by liver cells damaged by chronic inflammation, viral transactivation, and/or the regenerative response of the liver to cell loss (4). It has been reported that *IGF2* P1 promoter activity is absent in 70% of HCC cases (11). Uchida *et al.* (34) found evidence of switching from the adult P1 promoter to the fetal P2, P3 and P4 promoters of the *IGF2* gene in 17 out of 18 HCCs. These data suggest that most HCCs use the fetal promoters of *IGF2*. In the present study, we did not examine differential promoter usage for the *IGF2* gene; therefore, further studies are needed to clarify the relationship between imbalances in expression of *IGF2* and *H19* and *IGF2* promoter usage.

Much effort has been devoted to clarifying the mechanisms underlying altered regulation of transcription of *IGF2* and *H19* (6-10, 16). These studies have revealed that multiple systems are involved in transcriptional regulation of the two genes, including an imprinted control region (ICR) (8, 10), CCCTC-binding factor (CTCF) that binds to several sites in the unmethylated ICR (9), a differentially methylated region (DMR) silencer (7), and an *IGF2-H19* endodermal enhancer (16). Such data could improve our understanding of transcriptional regulation of genes at the imprinted region of 11p15 in somatic cells. However, the system of transcriptional control of these genes is likely to be complex due to variations in the transcription regulatory system used by various cell types, and it will be necessary to study how they are regulated in a tissue- or cancer-specific manner. In this regard, our present data show the significance of an imbalance in expression of *IGF2* and *H19* in HCC progression and may provide insights into the molecular role of *IGF2* and *H19* in HCC progression.

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References

1 Feinberg AP and Tycko B: The history of cancer epigenetics. Nat Rev Cancer *4*: 143-153, 2004.

2 Schwienbacher C, Gramantieri L, Scelfo R, Veronese A, Calin GA, Bolondi L, Croce CM, Barbanti-Brodano G and Negrini M: Gain of imprinting at chromosome 11p15: A pathogenetic mechanism identified in human hepatocarcinomas. Proc Natl Acad Sci USA *97*: 5445-5449, 2000.

3 Scelfo RA, Schwienbacher C, Veronese A, Gramantieri L, Bolondi L, Querzoli P, Nenci I, Calin GA, Angioni A, Barbanti-Brodano G and Negrini M: Loss of methylation at chromosome 11p15.5 is common in human adult tumors. Oncogene *21*: 2564-2572, 2002.

4 Thorgeirsson SS and Grisham JW: Molecular pathogenesis of human hepatocellular carcinoma. Nat Genet *31*: 339-346, 2002.

5 Poirier K, Chalas C, Tissier F, Couvert P, Mallet V, Carrie A, Marchio A, Sarli D, Gicquel C, Chaussade S, Beljord C, Chelly J, Kerjean A and Terris B: Loss of parental-specific methylation at the IGF2 locus in human hepatocellular carcinoma. J Pathol *201*: 473-479, 2003.

6 Brown KW, Villar AJ, Bickmore W, Clayton-Smith J, Catchpoole D, Maher ER and Reik W: Imprinting mutation in the Beckwith-Wiedemann syndrome leads to biallelic IGF2 expression through an H19-independent pathway. Hum Mol Genet *5*: 2027-2032, 1996

7 Constancia M, Dean W, Lopes S, Moore T, Kelsey G and Reik W: Deletion of a silencer element in Igf2 results in loss of imprinting independent of H19. Nat Genet *26*: 203-206, 2000.

8 Hark AT and Tilghman SM: Chromatin conformation of the H19 epigenetic mark. Hum Mol Genet *7*: 1979-1985, 1998.

9 Hark AT, Schoenherr CJ, Katz DJ, Ingram RS, Levorse JM and Tilghman SM: CTCF mediates methylation-sensitive enhancer-blocking activity at the H19/Igf2 locus. Nature *405*: 486-489, 2000.

10 Webber A, Ingram RS, Levorse JM and Tilghman SM: Location of enhancers is essential for the imprinting of H19 and Igf2 genes. Nature *391*: 711-715, 1998.

11 Li X, Nong Z, Ekstrom C, Larsson E, Nordlinder H, Hofmann WJ, Trautwein C, Odenthal M, Dienes HP, Ekstrom TJ and Schirmacher P: Disrupted IGF2 promoter control by silencing of promoter P1 in human hepatocellular carcinoma. Cancer Res *57*: 2048-2054, 1997.

12 Lee YI, Lee S, Das GC, Park US, Park SM and Lee YI: Activation of the insulin-like growth factor II transcription by aflatoxin B1 induced p53 mutant 249 is caused by activation of transcription complexes; implications for a gain-of-function during the formation of hepatocellular carcinoma. Oncogene *19*: 3717-3726, 2000.
13 Nardone G, Romano M, Calabro A, Pedone PV, de Sio I, Persico M, Budillon G, Bruni CB, Riccio A and Zarrilli R: Activation of fetal promoters of insulin-like growth factors II gene in hepatitis C virus-related chronic hepatitis, cirrhosis, and hepatocellular carcinoma. Hepatology *23*: 1304-1312, 1996.
14 Sohda T, Iwata K, Soejima H, Kamimura S, Shijo H and Yun K: *In situ* detection of insulin-like growth factor II (IGF2) and H19 gene expression in hepatocellular carcinoma. J Hum Genet *43*: 49-53, 1998.
15 Takeda S, Kondo M, Kumada T, Koshikawa T, Ueda R, Nishio M, Osada H, Suzuki H, Nagatake M, Washimi O, Takagi K, Takahashi T, Nakao A and Takahashi T: Allelic-expression imbalance of the insulin-like growth factor 2 gene in hepatocellular carcinoma and underlying disease. Oncogene *12*: 1589-1592, 1996.
16 Vernucci M, Cerrato F, Besnard N, Casola S, Pedone PV, Bruni CB and Riccio A: The H19 endodermal enhancer is required for Igf2 activation and tumor formation in experimental liver carcinogenesis. Oncogene *19*: 6376-6385, 2000.
17 Kim KS and Lee YI: Biallelic expression of the H19 and IGF2 genes in hepatocellular carcinoma.Cancer Lett *119*: 143-148, 1997.
18 Iizuka N, Oka M, Yamada-Okabe H, Mori N, Tamesa T, Okada T, Takemoto N, Tangoku A, Hamada K, Nakayama H, Miyamoto T, Uchimura S and Hamamoto Y: Comparison of gene expression profiles between hepatitis B virus- and hepatitis C virus-infected hepatocellular carcinoma by oligonucleotide microarray data on the basis of a supervised learning method. Cancer Res *62*: 3939-3944, 2002.
19 Iizuka N, Oka M, Yamada-Okabe H, Nishida M, Maeda Y, Mori N, Takao T, Tamesa T, Tangoku A, Tabuchi H, Hamada K, Nakayama H, Ishitsuka H, Miyamoto T, Hirabayashi A, Uchimura S and Hamamoto Y: Oligonucleotide microarray for prediction of early intrahepatic recurrence of hepatocellular carcinoma after curative resection. Lancet *361*: 923-929, 2003.
20 Iizuka N, Oka M, Yamada-Okabe H, Mori N, Tamesa T, Okada T, Takemoto N, Hashimoto K, Tangoku A, Hamada K, Nakayama H, Miyamoto T, Uchimura S and Hamamoto Y: Differential gene expression in distinct virologic types of hepatocellular carcinoma: association with liver cirrhosis. Oncogene *22*: 3007-3014, 2003.
21 Okada T, Iizuka N, Yamada-Okabe H, Mori N, Tamesa T, Takemoto N, Tangoku A, Hamada K, Nakayama H, Miyamoto T, Uchimura S, Hamamoto Y and Oka M: Gene expression profile linked to p53 status in hepatitis C virus-related hepatocellular carcinoma. FEBS Lett *555*: 583-590, 2003.
22 Iizuka N, Oka M, Yamada-Okabe H, Hamada K, Nakayama H, Mori N, Tamesa T, Okada T, Takemoto N, Matoba K, Takashima M, Sakamoto K, Tangoku A, Miyamoto T, Uchimura S and Hamamoto Y: Molecular signature in three types of hepatocellular carcinoma with different viral origin by oligonucleotide microarray. Int J Oncol *24*: 565-574, 2004.
23 Brazma A, Hingamp P, Quackenbush J, Sherlock G, Spellman P, Stoeckert C, Aach J, Ansorge W, Ball CA, Causton HC, Gaasterland T, Glenisson P, Holstege FC, Kim IF, Markowitz V, Matese JC, Parkinson H, Robinson A, Sarkans U, Schulze-Kremer S, Stewart J, Taylor R, Vilo J and Vingron M: Minimum information about a microarray experiment (MIAME)-toward standards for microarray data. Nat Genet *29*: 365-371, 2001.
24 Iizuka N, Oka M, Hamamoto Y, Mori N, Tamesa T, Okada T, Takemoto N, Matoba K, Takashima M, Sakamoto K, Tangoku A, Miyamoto T, Uchimura S, Nakayama H, Hamada K and Yamada-Okabe H: Altered levels of cytochrome P450 genes in hepatitis B or C virus-infected liver identified by oligonucleotide microarray. Cancer Genom Proteom *1*: 53-58, 2004.
25 Parkin DM, Bray FI and Devesa S: Cancer burden in the year 2000. The global picture. Eur J Cancer *37*: S4-S66, 2001.
26 Tepetes KN, Tsamandas AC, Felekouras ES, Salakou SG and Karavias DD: Conversion of unresectable hepatoma to resectable. Report of a case and review of the literature. Hepatogastroenterology *47*: 1105-1109, 2000.
27 Bassanello M, Cillo U, Vitale A, Lumachi F, Ciarleglio FA, Boccagni P, Brolese A, Zanus G, D'Amico F, Senzolo M and D'Amico DF: Multimodal approach and its impact on survival for patients with hepatocellular carcinoma. Anticancer Res *23*: 4047-4053, 2003.
28 Hsu HC, Tseng HJ, Lai PL, Lee PH and Peng SY: Expression of p53 gene in 184 unifocal hepatocellular carcinomas: association with tumor growth and invasiveness. Cancer Res *53*: 4691-4694, 1993.
29 Iizuka N, Oka M, Noma T, Nakazawa A, Hirose K and Suzuki T: NM23-H1 and NM23-H2 messenger RNA abundance in human hepatocellular carcinoma. Cancer Res *55*: 652-657, 1995.
30 Iizuka N, Mori N, Tamesa T, Tangoku A and Oka M: Telomerase activity and Nm23-H2 protein expression in hepatocellular carcinoma. Anticancer Res *23*: 43-47, 2003.
31 Poon RT, Lau CP, Ho JW, Yu WC, Fan ST and Wong J: Tissue factor expression correlates with tumor angiogenesis and invasiveness in human hepatocellular carcinoma. Clin Cancer Res *9*: 5339-5345, 2003.
32 Tannapfel A, Anhalt K, Hausermann P, Sommerer F, Benicke M, Uhlmann D, Witzigmann H, Hauss J and Wittekind C: Identification of novel proteins associated with hepatocellular carcinomas using protein microarrays. J Pathol *201*: 238-249, 2003.
33 Saito Y, Kanai Y, Nakagawa T, Sakamoto M, Saito H, Ishii H and Hirohashi S: Increased protein expression of DNA methyltransferase (DNMT) 1 is significantly correlated with the malignant potential and poor prognosis of human hepatocellular carcinomas. Int J Cancer *105*: 527-532, 2003.
34 Uchida K, Kondo M, Takeda S, Osada H, Takahashi T, Nakao A and Takahashi T: Altered transcriptional regulation of the insulin-like growth factor 2 gene in human hepatocellular carcinoma. Mol Carcinog *18*: 193-198, 1997.

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