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.
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).

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

<table>
<thead>
<tr>
<th>Factors</th>
<th>Imbalanced type (n=19)</th>
<th>Balanced type (n=40)</th>
<th>P value</th>
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<tbody>
<tr>
<td>Sex</td>
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<tr>
<td>Male (n=43)</td>
<td>13</td>
<td>30</td>
<td>p=0.595*</td>
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<td>Female (n=16)</td>
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<tr>
<td>Age (year)</td>
<td>65.2±1.6</td>
<td>61.8±1.4</td>
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<td>Virus type</td>
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<td>B type (n=11)</td>
<td>4</td>
<td>7</td>
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<tr>
<td>Non-B, non-C (n=8)</td>
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<td>Tumor size (cm)</td>
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<td>3.7±0.4</td>
<td>p=0.092**</td>
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<tr>
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<td>pTNM Stage</td>
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<tr>
<td>(-) (n=38)</td>
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</tr>
<tr>
<td>(+) (n=21)</td>
<td>9</td>
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*pChi-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.
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 livers. 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.

Acknowledgements

Supported in part by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan (No.12671230).

References

Iizuka et al: IGF2 and H19 in HCC


Received May 24, 2004
Accepted September 15, 2004