ORIGINAL ARTICLE

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In situ detection of insulin-like growth factor II (*IGF2*) and *H19* gene expression in hepatocellular carcinoma

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Abstract To assess the relationship between insulin-like growth factor II (*IGF2*) and *H19* gene expression at the cellular level, we have examined the distribution of *IGF2* and *H19* mRNA by means of in situ hybridization in hepatic malignancies consisting of hepatocellular carcinoma (HCC), cholangiocellular carcinoma (CCC), and metastatic liver cancer (MLC). In HCC, 15 of 27 tumors (56%) and 11 of 27 tumors (41%) demonstrated increased *IGF2* and *H19* gene expression, respectively. Of 16 HCCs with increased expression of either *IGF2* or *H19*, 10 tumors coexpressed both transcripts at comparable levels. Moreover, the spatiotemporal distribution and the cellular localization of the two gene transcripts were almost identical, suggesting the presence of a reciprocal relation between *IGF2* and *H19*. In addition, 5 HCCs showed increased *IGF2* expression without concomitant *H19* expression, whereas 1 HCC showed increased *H19* expression without *IGF2* transcripts. However, 11 HCCs showed no *IGF2* or *H19* expression. On the other hand, neither *IGF2* transcripts nor *H19* transcripts were detected in 2 CCCs or 10 MLCs studied. The data suggest that *IGF2* and/or *H19* gene expression may be characteristic of some HCCs.

Key words *IGF2* *H19* · Gene · Hepatocellular carcinoma · In situ hybridization

Introduction

Insulin-like growth factor II (*IGF2*), whose gene is located on chromosome 11p15.5, is a growth factor believed to be important in fetal growth and development (Moses et al.

1980; Brown et al. 1986; Humbel 1990). The human *IGF2* gene is one of a dozen genes which are subject to genomic imprinting, with the paternal allele expressed and the maternal allele silent. The *IGF2* gene is a complex transcriptional unit that contains four different promoters (P1–P4) (Sussenbach et al. 1991; van Dijk et al. 1991). During fetal life, a high level of *IGF2* transcript is expressed mainly from the P2–P4, fetal-type promoters, and the resulting *IGF2* serves as a growth factor in several embryonic tissues including fetal liver. In contrast, adult liver demonstrates loss of imprinting (LOI) of *IGF2* which is derived from P1, leading to biallelic *IGF2* expression, while *IGF2* transcripts derived from P2–P4 show markedly decreased expression, although they remain monoallelically expressed (Vu and Hoffman 1994; Ekström et al. 1995).

It has been reported that *IGF2* plays a role in the pathogenesis of a variety of neoplasms (LeRoith et al. 1995). Since increased expression of *IGF2* in hepatocellular carcinoma (HCC) was originally described by Cariani et al. (1988), several investigators have reported that *IGF2* may play a role in the development of HCC (D'Arville et al. 1991; Lamas et al. 1991; Sohda et al. 1996).

The *H19* gene, which is located 200kb downstream of *IGF2*, has also been implicated in tumor development and is believed to function as a tumor suppressor (Zhang and Tycko 1992; Hao et al. 1993). *H19* has also been shown to be imprinted, with the maternal allele being expressed and the paternal silent, in contrast to *IGF2*, for which the reverse situation applies. This unique reciprocal relationship, along with the observation that *IGF2* and *H19* genes are extensively coexpressed during human embryonic/fetal development (Ohlsson et al. 1994), has led to the so-called enhancer competition model hypothesis. This model proposes that there exists a regulatory element near the *IGF2*/*H19* loci that acts as a switch to allow either *IGF2* or *H19* expression (Pfeifer and Tilghman 1994).

It was recently reported that the expression of *IGF2* and *H19* in HCC were related to each other (Takeda et al. 1996; Li et al. 1997). However, it remains unclear whether high expression of *IGF2* and *H19* occurs within a single cell. In this study, to assess the relationship between *IGF2* and *H19*

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gene expression at the cellular level in HCC, we carried out in situ hybridization and compared the level and the localization of *H19* transcripts. In addition, we examined expression of these transcripts in other liver malignancies: cholangiocellular carcinoma (CCC) and metastatic liver cancer (MLC). The results demonstrated that increased levels of *IGF2* expression were detected in approximately half of the HCCs, and the distribution and the intensity of *IGF2* transcripts showed a close resemblance to those of *H19*, whereas other liver cancers showed no detectable levels of *IGF2* or *H19* gene expression.

Materials and methods

Tissue specimens

The present study comprised 27 HCCs, 2 CCCs, and 10 MLCs, all of which were from an autopsy file of the Fukuoka University Hospital. The diagnosis was based upon histological findings and clinical examination. Routine staining was performed to evaluate histological type. HCC was histologically graded according to the classification of Edmondson and Steiner (1954), and the results are summarized in Table 1. Of 27 HCCs, 14 were positive for hepatitis C virus antibody (HCVAb) only, 6 were positive for hepatitis B surface antigen (HBsAg) only, 1 was positive for both

HBsAg and HCVAb, and 6 were negative for both HBsAg and HCVAb (Table 1).

All liver samples were fixed in buffered 10% formalin and embedded in paraffin. Serial 5-mm-thick sections were cut and mounted on slides coated with 2% 3-aminopropyltriethoxysilane. Slides were then heated at 60°C for 2h and stored at room temperature until use.

RNA probes

The human *IGF2* was a 559-bp fragment corresponding to nucleotides 256–815 obtained from cDNA of *phigh2* cloned into pGEM-3. The *H19* clone correspond to a *EcoRI-SacI* fragment of human *H19* cDNA cloned into pGEM-7ZF(+). Sense and antisense RNA probes were transcribed from linearized templates using SP6 and T7 RNA polymerases (Promega, Madison, WI, USA), and radiolabeling with [³⁵S]-CTP (Amersham, Buckinghamshire, UK).

In situ hybridization

In situ hybridization analyses were performed according to the methods previously described (Wilkins et al. 1989). Briefly, hybridization was carried out in a solution containing 50% formamide, 2 × SSC, 0.15 M NaCl, 0.2 mg/ml *Escherichia coli* tRNA, 1 mg/ml degraded herring sperm DNA, 0.1 mg/ml bovine serum albumin, 10% polyethylene

Table 1 Clinical, histological and in situ hybridization summary of hepatocellular carcinomas

Cases	Age/Sex	Virus markers	Non-T	T ^a	<i>IGF2/H19</i>
1	69/M	HCVAb(+)	LC	II	+++ / +++
2	64/M	HCVAb(+)	LC	I	++ / ++
3	26/F	HBsAg(+)	LC	III	++ / ++
4	17/F	HBsAg(-), HCVAb(-)	Normal	II	++ / ++
5	49/M	HBsAg(+)	CAH	III	++ / -
6	57/M	HCVAb(+)	LC	I	+/+
7	59/M	HCVAb(+)	LC	II	+/+
8	68/M	HCVAb(+)	LC	I	+/+
9	68/M	HCVAb(+)	LC	II	+/+
10	44/M	HBsAg(+)	CAH	III	+/+
11	68/M	HBsAg(-), HCVAb(-)	LC	II	+/+
12	68/M	HCVAb(+)	LC	I	+/+
13	61/M	HCVAb(+)	LC	II	+/+
14	60/M	HCVAb(+)	LC	II	+/+
15	75/F	HCVAb(+)	LC	III	+/+
16	75/M	HBsAg(-), HCVAb(-)	LC	II	- / +
17	60/M	HCVAb(+)	LC	II	- / -
18	66/F	HCVAb(+)	LC	I	- / -
19	62/M	HCVAb(+)	LC	II	- / -
20	59/M	HCVAb(+)	LC	II	- / -
21	51/M	HBsAg(+)	LC	III	- / -
22	40/F	HBsAg(+)	LC	II	- / -
23	45/M	HBsAg(+)	LC	I	- / -
24	87/M	HBsAg(-), HCVAb(-)	LC	II	- / -
25	62/M	HBsAg(-), HCVAb(-)	LC	I	- / -
26	67/M	HBsAg(-), HCVAb(-)	LC	III	- / -
27	77/M	HBsAg(+), HCVAb(+)	LC	I	- / -

^a Edmondson and Steiner's classification.

Non-T, non-tumorous liver; T, tumorous lesion; LC, liver cirrhosis; CAH, chronic active hepatitis.

+++ , Strong positivity; ++ , moderate; + , weak; - , slight or nil.

glycol, 0.2M dithiothreitol, and [35 S]-CTP-labeled anti-sense or sense RNA probe (40,000 cpm/ml) at 50°C overnight. After hybridization, the slides were washed in 50% formamide, 2 × SSC at 52°C and in 2 × SSC at room temperature. The slides were then incubated in RNase A solution at 37°C for 30 min. For autoradiography, slides were dipped into nuclear emulsion (Amersham) and exposed in light-tight boxes at 4°C for 2 weeks. After development, the slides were counterstained with hematoxylin-eosin.

Results

In situ mRNA hybridization in HCC

Among the 27 HCCs examined by in situ hybridization, increased expression of the *IGF2* gene was observed in 15 HCC cases (56%), which consisted of 10 of 14 cases associated with hepatitis C virus (HCV), 3 of 6 cases associated with hepatitis B virus (HBV), and 2 of 6 cases negative for both HBV and HCV, and did not include the single case with both HBV and HCV. On the other hand, an increased level of *H19* transcripts was detected in 11 (41%) of 27

HCCs. These 11 tumors included 6 of 14 cases positive for HCV, 2 of 6 positive for HBV, and 3 of 6 negative for both HBV and HCV, and also excluded the case with both HBV and HCV. Collectively, 16 of 27 HCCs showed increased expression of either the *IGF2* or the *H19* gene. Among these 16 HCCs, 10 tumors showed both transcripts, while 5 produced only *IGF2* transcripts, and 1 had only *H19* transcripts. There appeared to be no obvious, statistically significant correlations among tumors with or without *IGF2* and/or *H19* expression, in terms of histological and clinical parameters.

With respect to the spatial or cellular localization, *IGF2* and *H19* transcripts were detected specifically in the cytoplasm of HCC cells. Significant levels of hybridization signal were detected in less-well-differentiated HCC cells which were located in the periphery of HCC tumor nodules, whereas well-differentiated tumor cells in the central area showed no detectable level of transcripts (Fig. 1). On the other hand, regenerative hepatocytes in cirrhotic liver from nontumorous areas contained low levels of transcripts. All of the 10 tumors with increased *IGF2* and *H19* transcripts showed almost identical cellular localization of the two transcripts, strongly suggesting that the tumor cells co-express both transcripts. Furthermore, the levels of the two gene transcripts in these 10 HCCs appeared to be closely and positively correlated (Table 1).

Fig. 1 In situ hybridization analyses of *IGF2* and *H19* genes in hepatocellular carcinoma (HCC). **a** and **c** are bright field photomicrographs; **b** and **d** are the respective corresponding dark field photomicrographs. High levels of *IGF2* transcripts were observed in the cytoplasm of HCC cells that are located at the periphery of tumor nodules (**a** and **b**). A

serial section demonstrates that increased levels of *H19* transcripts were also detected in the HCC cells that are located at periphery of tumor nodules (**c** and **d**). The localization pattern of the *H19* gene was almost identical to that of *IGF2*. Bar, 200 nm

Table 2 Clinical and in situ hybridization summary of other liver cancers

Cases	Age/Sex	Origins	Histology	<i>IGF2/H19</i>
1	66/M	Lung	Squamous cell carcinoma	-/-
2	61/M	Lung	Adenocarcinoma	-/-
3	54/F	Uterine cervix	Squamous cell carcinoma	-/-
4	47/F	Uterine cervix	Adenosquamous cell carcinoma	-/-
5	69/M	Kidney	Renal cell carcinoma	-/-
6	54/F	Iliac bone	Fibroblastic osteosarcoma	-/-
7	57/M	Hypopharynx	Squamous cell carcinoma	-/-
8	84/M	Stomach	Adenocarcinoma	-/-
9	73/F	Pancreas	Tubular adenocarcinoma	-/-
10	67/M	Colon	Adenocarcinoma	-/-
11	50/M	Liver	Cholangiocellular carcinoma	-/-
12	78/F	Liver	Cholangiocellular carcinoma	-/-

-, slight or nil.

Expression of *IGF2* and *H19* in other liver cancers

Tumors from 10 MLCs were also included in this study. The primary sites are shown in Table 2. Neither *IGF2* nor *H19* transcripts were expressed in liver metastases or primary tumors, while low levels of both transcripts were detected in the hepatocytes from nonneoplastic lesions. Similarly, in 2 CCCs, neither *IGF2* nor *H19* transcripts were detected (data not shown).

Discussion

We have examined the expression patterns of the *IGF2* and *H19* genes in HCC by means of in situ hybridization. It is generally known that the formalin-fixed section technique is also available to perform in situ mRNA hybridization. However, to avoid the degradation of RNA by endogenous RNase, we have used liver sections which were fixed in formalin within 3 h of cardiac arrest. Moreover, we confirmed the specificity by: (1) pretreatment of tissue sections with RNase A (100 mg/ml) for 1 h at 37°C before hybridization; (2) hybridization with sense probes; and (3) hybridization of fetal liver samples as positive controls (data not shown).

HCC is known to be one of the neoplasms which produce IGF2. Nardone et al. (1996) reported *IGF2* expression in HCCs associated with HCV. In addition, Su et al. (1994) reported a role for IGF2 in HCC associated with HBV, i.e., products of the integrated hepatitis B *x* gene may activate the *IGF2* gene. The present study found increased *IGF2* expression in 56% of HCCs. The increased expression of *IGF2* is envisaged to be advantageous for tumor growth. Most of the growth factors produced by tumor cells are thought to promote the growth of the tumor itself (Heldin and Westermark 1984; Sporn and Roberts 1985). In the present study, the high level of hybridization signal was detected in less-well-differentiated tumor cells but not in well-differentiated cells, suggesting that IGF2 may act as an autocrine or paracrine growth factor. This notion is supported by our recent observation in that IGF2 protein and Ki-67 antigen, a proliferation marker, are codetected in

less-well-differentiated tumor cells (Sohda et al. 1997). We have also previously demonstrated that in HCC, the increased *IGF2* mRNA is transcribed largely from the fetal type promoter, P3, rather than the adult type promoter, P1, and that the level of *IGF2* mRNA is comparable to that of IGF2 protein in tumor cells, indicating that IGF2 production is primarily regulated at the gene transcriptional level without significant posttranscriptional modification (Sohda et al. 1996). These reports by us and others suggest that IGF2 may play a primary role as an autocrine growth factor in HCC.

The *H19* gene, located telomeric to *IGF2* on chromosome 11p15.5, which is frequently involved in loss of heterozygosity (LOH) in a variety of cancers, including HCC (Viel et al. 1992; Winqvist et al. 1993; Baffa et al. 1996; Besnard-Guérin et al. 1996), is subject to genomic imprinting with maternal gene expression only. *H19* transcripts were detected in 41% of HCCs. Tumors expressing the two transcripts showed similar cellular localization patterns for *H19* and *IGF2* transcripts, a result consistent with the enhancer competition model. According to the model, the regulatory units telomeric to *IGF2* and *H19* genes control a switch for either *IGF2* or *H19* expression, thus leading to reciprocal expression of the two genes (Pfeifer and Tilghman 1994). This model appears to fit well in endodermal tissues such as liver, or in the endomesodermal mixed tissue including gut, kidney, and lung. In other tissues, such as placenta, skeletal muscle, and brain, the model does not fit, suggesting the involvement of other factors in the control of *H19* and *IGF2* expression. The model gains support from the observation that biallelic expression of *IGF2* appears to be associated with decreased expression of *H19* in childhood tumors such as Wilms' tumor and hepatoblastoma, suggesting that *H19* plays a role in tumor suppression as a *cis*-acting repressor of *IGF2* (Li et al. 1995; Steenman et al. 1994; Moulton et al. 1994). We have reported that early-stage placentae showed biallelic *H19* and monoallelic *IGF2* expression in the same placental trophoblasts (Jinno et al. 1995).

Our present study indicated that neither *IGF2* nor *H19* transcripts were detected in 11 of 27 HCCs and that comparable levels of the two gene transcripts were seen in 10 tumors (37%). On the other hand, 5 tumors (18.5%) were

associated with the upregulation of *IGF2* and the downregulation of *H19*. One tumor (4.5%) was associated with the downregulation of *IGF2* and the upregulation of *H19*. Therefore, our data suggest that, in contrast to Wilms' tumor, in HCC, *H19* may have a limited role, and that gene activation of *IGF2* and *H19* may be involved in the complicated mechanism of hepatocarcinogenesis.

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