SMAD5 Gene Expression, Rearrangements, Copy Number, and Amplification at Fragile Site FRA5C in Human Hepatocellular Carcinoma

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Abstract

Signaling by the transforming growth factor (TGF)-family members is transduced from the cell surface to the nucleus by the Smad group of intracellular proteins. Because we detected alterations on the long arm of chromosome 5, we examined the status of the *SMAD5* gene in human hepatocellular carcinoma (HCC) cell lines and primary HCC. In 16 cell lines, chromosome alterations of chromosome 5 were observed in nine cell lines by fluorescence *in situ* hybridization (FISH), and an increase in *SMAD5* gene copy number relative to the ploidy level was found in eight lines. The breakpoints in unbalanced translocations and deletions frequently occurred near the SMAD5 locus, but apparently did not cause loss of SMAD5. In one cell line, where comparative genomic hybridization showed DNA copy number gain confined to the region 5q31, we detected by FISH high-level amplification of the *SMAD5* gene located within the fragile site FRA5C. Semiquantitative polymerase chain reaction did not reveal changes in SMAD5 DNA levels in 15 of 17 primary HCC specimens. In 17 HCC cell lines, SMAD5 mRNA levels were either maintained or upregulated by an increase in gene dosage or another mechanism. Collectively, our results show that SMAD5 undergoes copy number gain and increased expression, rather than loss of expression, and therefore suggest that this gene does not act as a tumor-suppressor gene in HCC. The Hep-40 HCC cell line with high-level amplification and significant overexpression of SMAD5 may be useful in studying the interaction of SMAD5 with other genes.

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Introduction

Chromosomal translocations, inversions, deletions, and amplifications contribute to the pathogenesis of human cancer by affecting the expression of genes involved in regulating cell growth [1–3]. Our characterization of recurrent chromosome changes in human hepatocellular carcinoma (HCC) at the molecular level is directed toward detecting alterations of known genes and isolating new genes that may be implicated in the development of liver neoplasia. From one recurrent region of deletion on the short arm of chromosome 8 in HCC, we isolated the *DLC-1* gene and recently showed that it operates as a tumor suppressor in human breast cancers [4,5]. Among known genes, we detected the deletion and downregulation of the tumor-suppressor gene *FHIT* at 3p and the amplification of the *EMS1* oncogene at 11q, suggesting that alterations of these genes are pathologically relevant to HCC and might be useful markers for the early detection and prognosis of the disease [6,7].

The transforming growth factor (TGF)-β family of cytokines regulates a number of cellular processes, including proliferation, apoptosis, differentiation, and pattern formation [8]. Signaling by members of the TGF-β family is transduced from the cell surface to the nucleus by the Smad group of intracellular proteins. Binding to receptors for the TGF-β family members leads to the phosphorylation and activation of the receptor-regulated Smads, which include Smads 1, 2, 3, 5, and 8 [8,9]. The activated Smads bind to the common mediator Smad4, forming a complex that translocates to the nucleus and regulates the transcription of target genes. TGF-β inhibits the growth of many epithelial cells, and during the neoplastic process, many carcinomas acquire resistance to the antiproliferative signals of TGF-β [10]. In some tumors, the loss of TGF-β responsiveness is associated with inactivation of one of the components of the TGF-β signaling pathway, and mutations in the *SMAD4* and *SMAD2* genes have been found in several types of cancer [10,11].

Our previous comparative genomic hybridization (CGH) studies and our ongoing spectral karyotyping (SKY) analyses

of HCC cell lines showed both DNA copy number gains and losses of the long arm of chromosome 5 [12]. The minimal region of gain at 5q31 overlaps with the location of the common fragile site (FS) FRA5C and the locus of the *SMAD5* gene. The role of the Smad proteins in cancer and the association of FS with sites of chromosomal breakage in tumors prompted us to examine whether alterations in the *SMAD5* gene were present in human HCC and whether these were associated with the region of FRA5C.

Materials and Methods

HCC Cell Lines

Huh-6, Huh-7, HLF, HLE, Hep3B, Hep-40, HepG2, Focus, 7703K, SK-Hep-1, PLC/PRF/5, and the six SNU lines were obtained from the American Type Culture Collection (Rockville, MD), or kindly provided by Dr. Curtis Harris (National Cancer Institute, Bethesda, MD). These cell lines were previously analyzed by CGH and their hepatitis B virus (HBV) status was known [12]. Five cell lines (7703, SK-Hep-1, HepG2, Huh-7, HLF, and HLE) were negative for HBV [12]. Cells were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum and antibiotics.

Northern Blot Analysis

Total RNA was extracted from HCC cell lines using Trizol reagent (Invitrogen/Life Technologies, Carlsbad, CA). Normal human liver total RNA was purchased from Stratagene (La Jolla, CA; cat no. 735018). Twenty-microgram aliquots of each RNA sample were denatured in 50% formamide/6.5% formaldehyde and resolved on 1% agarose/4-morpholine-propanesulfonic acid/formaldehyde gels and transferred to nylon membranes. A human SMAD5 cDNA clone (no. 262686; IMAGE Consortium, http://image.llnl.gov) was purchased from the American Type Culture Collection (Manassas, VA). A 1-kb *Hin*dIII fragment at the 3' end of the insert (corresponding to nt 984–1969 of GenBank U59913) was labeled with [α-^{32}P]dCTP by the random primer method and hybridized to Northern blots as previously described [5]. As a control for loading, the blots were rehybridized to a polymerase chain reaction (PCR)–generated probe for the murine ribosomal protein L19 cDNA (nt 120–495 of GenBank NM_009078). The blots were exposed to X-ray film, the autoradiograms were scanned on an HP Scanjet 4400c (Hewlett Packard, Palo Alto, CA), and the SMAD5 and L19 bands were quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Southern Blot Hybridization

Genomic DNA was purified from HCC cell lines by a modification of the original method [13]. Genomic DNA from normal human fibroblasts was obtained from the Coriell Institute for Medical Research (Camden, NJ; cat no. NA00726). Five-microgram aliquots of DNA were digested with *Hin*dIII and fractionated on a 1% agarose/TAE gel. The DNA was blotted onto a nylon membrane and hybridized to the SMAD5 cDNA probe as indicated above. As a control for

the copy number of chromosome 5, the blot was rehybridized to a ^{32}P-labeled, PCR-generated probe for the occludin gene (nt 179471–180050 of GenBank AC044797) located at 5q12.

Semiquantitative PCR Analysis

Genomic DNA samples from 17 primary HCC and matching normal liver tissues [4] were subjected to multiplex PCR analysis. Primer pairs from the 3' UTRs of the human SMAD5 cDNA (5'-CCAGTTCAGAAATTTGGCATTG and 5'-TGGTGGGACAAAGAAA CTGG; nt 1761–1782 of GenBank U59913) and of the human occludin cDNA (5'-GAAGC-CAAACCTCTGTGAGC and 5'-GAAGACATCGTCTGGG-GTGT; nt 1825–2053 of GenBank U49184), an internal control, were selected using the Primer3 program of the Whitehead Institute/MIT Center for Genome Research (http://www-genome.wi.mit.edu). Amplifications were performed using 50 ng of genomic DNA, 10 pmol of each primer, 1.0 U of Taq DNA polymerase (Promega, Madison, WI), 300 μM dNTPs, 1.5 mM MgCl$_2$, and 1/10 vol of 10 $\times$ PCR buffer (10 mM Tris–HCl, pH 9.0, 50 mM KCl, and 0.1% Triton X-100) in a final reaction volume of 30 μl. The optimized conditions for quantitative PCR conditions were as follows: an initial denaturation for 5 minutes at 95°C followed by 25 cycles of 94°C for 50 seconds, 60°C for 40 seconds, 72°C for 50 seconds, and a terminal extension for 5 minutes at 72°C. The PCR products were loaded on 6% acrylamide/TBE precast gels (BMA, Rockland, ME) and electrophoresed at 180 V for 1 hour in 0.5 $\times$ TBE buffer. The PCR products were visualized by ethidium bromide staining and photographed under UV transillumination. The individual bands were quantified by densitometry (Kodak Gel Logic 100 Image System, Rochester, NY) and the relative intensities of the SMAD5 and occludin bands were calculated.

Induction of FS FRA5C

For the expression of FRA5C, synchronized cultures of human lymphocytes derived from two healthy donors were

Table 1. Number of SMAD5 FISH Signals and of Normal Chromosome 5 in HCC Cell Lines.

Cell Line	SMAD5	Chromosome 5
PLC/PRF/5	2	2
Focus	3	2
HepG2	2	2
Hep3B	2	2
Hep-40*	2	2
HLE	4	2
HLF	3	1
SNU182	4	4
SNU387	3	3
SNU398	3	1
SNU423	5	5
SNU449	2	2
SNU475	3	1
7703K	4	1
SK-Hep-1	3	3
Huh-7	3	2

*Besides fluorescent SMAD5 signals on normal chromosomes 5, Hep-40 cells carry multiple additional SMAD5 signals located on rearranged derivative chromosomes that contain materials from chromosome 5.
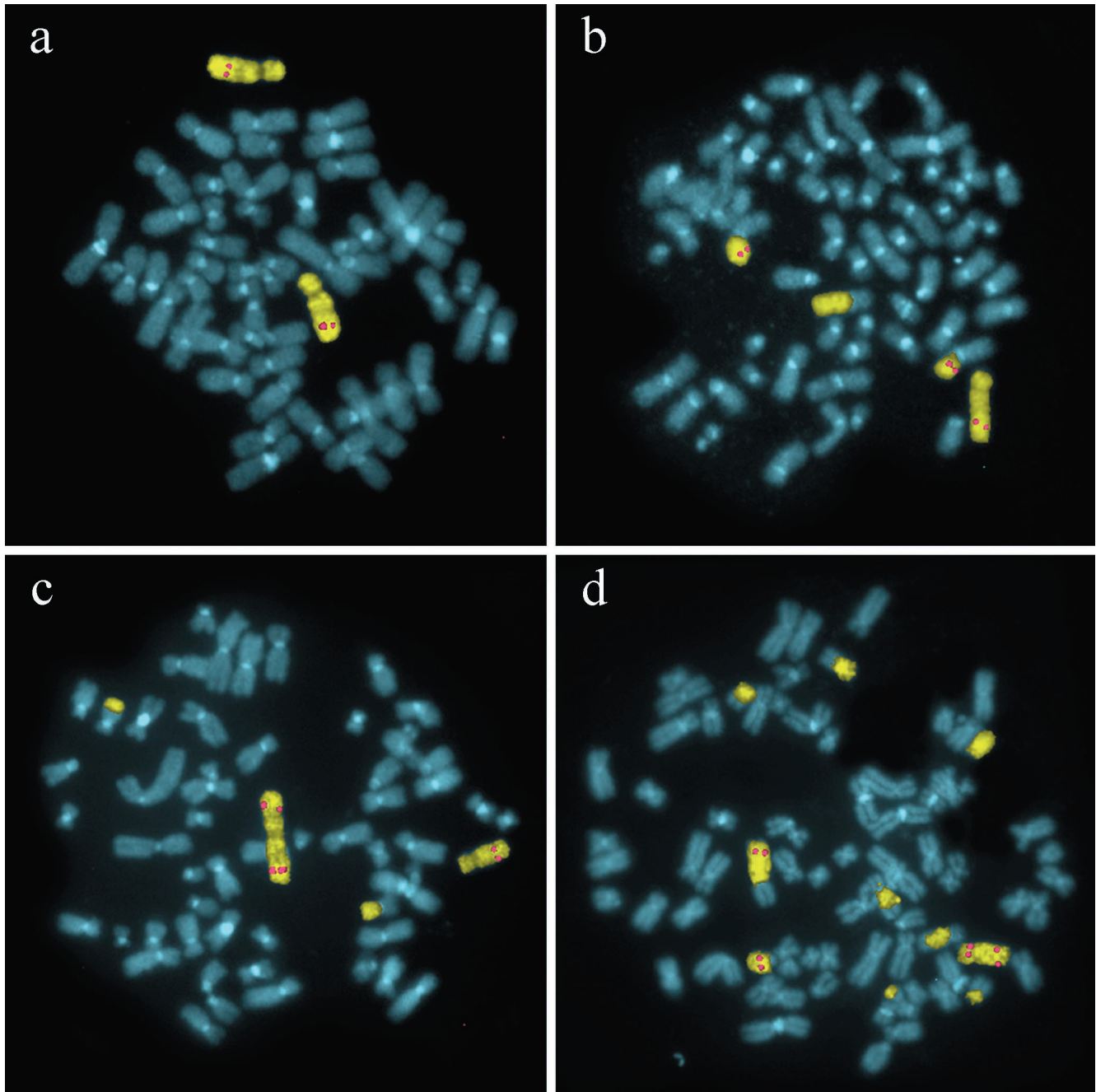
Figure 1. *Two-color FISH detection of chromosome 5 (yellow, biotin-labeled) and the SMAD5 gene (red, digoxigenin-labeled) in metaphase chromosome spreads from human HCC cell lines SNU449 (a), SNU398 (b), HLF (c), and 7703K (d).*

used. Cells were treated with aphidicolin (apc) (0.2–0.4 μM; Sigma, St. Louis, MO) in DMSO or in ethanol (0.2%) for 26 hours, and subsequently exposed to caffeine (2.2 μM; Sigma) for 6 hours prior to chromosome preparation as previously described [14]. Metaphase spreads were prepared after 3 to 4 hours of colcemid (50 μg/ml) treatment by standard KCl hypotonic treatment, acetic acid–methanol fixation, and air-dried slide preparation.

Molecular Cytogenetic Analysis
Chromosomes prepared from exponentially growing HCC cultures were used for fluorescence *in situ* hybridization

(FISH) and SKY. Chromosome preparations derived from synchronized normal human peripheral lymphocytes were used for CGH analysis of HCC genomic DNA. Metaphase spreads from synchronized, apc/caffeine–treated normal human peripheral lymphocytes were used for FISH visualization of SMAD5 signals relative to FRA5C. In all FISH experiments regardless of the target chromosome preparations, a P1-derived artificial chromosome (PAC) genomic clone containing the *SMAD5* gene labeled with digoxigenin, and biotinylated centromeric and whole chromosome 5 painting probes were used. The SMAD5 signals were localized on look-up table (LUT)-inverted and contrast-enhanced

digital images of DAPI-counterstained G-like banded chromosomes with approximately 400 band resolution. The procedures used for CGH, SKY, and FISH were previously described in detail [12,15,16].

Results

A recurrent CGH pattern of DNA copy number gains and losses of chromosome 5 was reported previously in HCC cell lines [12]. In addition, our previous chromosome painting and G-banding analysis as well as the ongoing SKY investigation of HCC cell lines showed a variety of changes involving chromosome 5q [16]. To determine the extent and nature of chromosome 5 reorganization and its impact on the copy number of the *SMAD5* gene, FISH was performed with a SMAD5 genomic probe and chromosome 5 painting probe in 16 HCC cell lines. All lines are aneuploid with a near-triploid or near-tetraploid chromosome number. In eight cell lines, an equal number of FISH signals for SMAD5 and normal chromosomes 5 were observed (Table 1). An example of a metaphase derived from SNU449 cells with normal chromosome 5 and signals for SMAD5 is shown in Figure 1a. In metaphases from the Focus, Hep-40, HLE, HLF, Huh-7, SNU398, SNU475, and 7703K cell lines, the signals for *SMAD5* gene exceeded the number of normal chromosome 5 and were also localized on abnormal chromosome 5. None of the cell lines had fewer SMAD5 signals than copies of chromosome 5 (Table 1).

Structural changes involving chromosome 5 consisted of interstitial deletions, unbalanced translocations, and the formation of isochromosomes containing the whole chromosome 5q or segments of chromosome 5q. Isochromosomes carrying SMAD5 signals were identified in HLF, HLE, and SNU423 cells, whereas deletions and translocations with the breakpoints near the SMAD5 locus as indicated by the FISH signal were found in Focus, Huh-7, SNU398, and 7703K cells. Representative examples of chromosome 5 alterations found in HCC cell lines are shown in Figure 1; a translocation in SNU398 cells with the breakpoint near SMAD5 locus (Figure 1b), an isochromosome 5q in HLF cells (Figure 1c), and multiple dispersed segments of chromosome 5, carrying SMAD5 signals in 7703K cells (Figure 1d), are shown.

In Hep-40 cells, one of four cell lines exhibiting DNA copy number gain in the distal region of chromosome 5q, a high-level SMAD5 amplification was detected by FISH with a genomic probe (Figure 2). In interphase nuclei, multiple SMAD5 signals were visible, and scoring of over a hundred nuclei gave an average of 13 to 17 signals per cell (Figure 2a). In all 50 metaphase spreads examined from Hep-40 cells, two pairs of SMAD5 signals were located on normal chromosome 5, and an orderly array of signals was clustered on an abnormal chromosome (Figure 2b). CGH profile showed that the region of gain was confined to a single band, 5q31, where the *SMAD5* gene is localized (Figure 2c). SKY analysis demonstrated that the abnormal chromosome carrying multiple copies of *SMAD5* gene is a complex rearrangement involving chromosomes 4, 5, 15, 18, and 19 (Figure 2d). The identified region of amplification (Figure 2d) was larger than the band 5q31, and had an abnormal
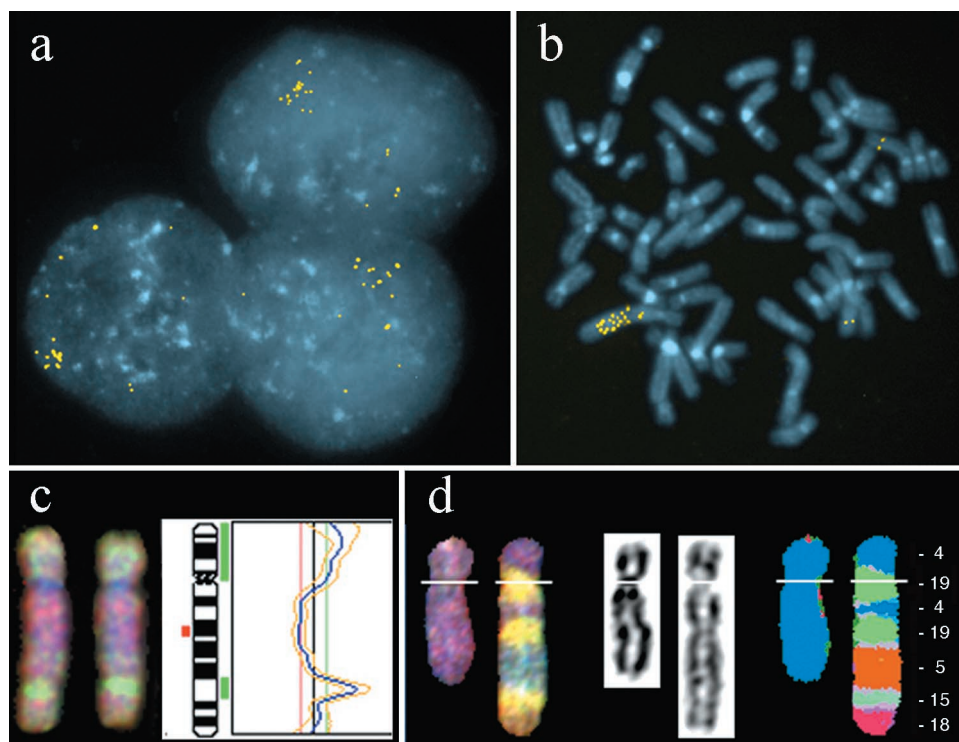

Figure 2. *Multiple SMAD5 signals in interphase nuclei from HCC cell line Hep-40 (a); an array of SMAD5 fluorescent signals localized on an abnormal chromosome containing an abnormally banded region (b); and FISH localization confirming local high-level amplification of the region of chromosome 5q31 harboring the SMAD5 gene as indicated by CGH (c). SKY analysis enabled the characterization of the abnormal chromosome harboring SMAD5 amplification (d) as a complex rearrangement involving chromosomes 4, 5, 15, 18, and 19.*
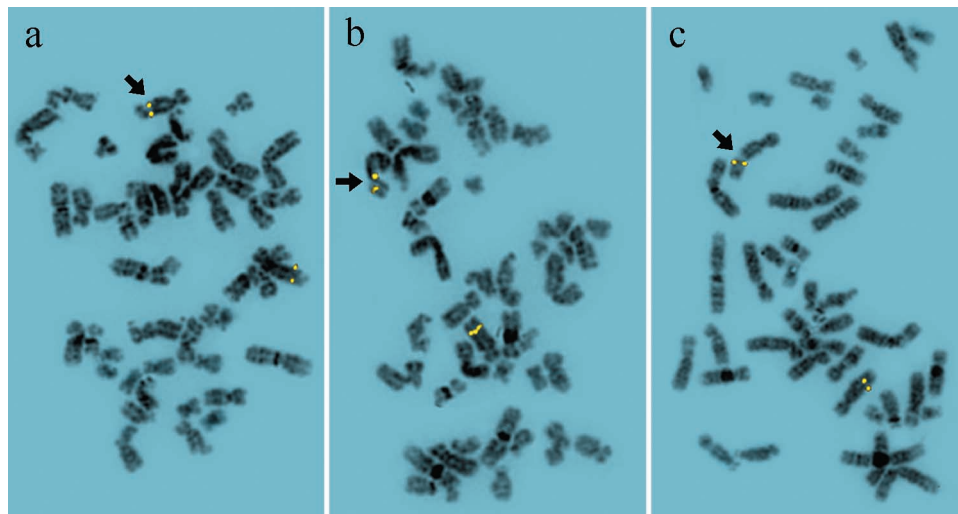
Figure 3. *FISH with the SMAD5 probe on chromosomes from apc-treated normal lymphocytes, localizing the SMAD5 signal within the domain of the FRA5C FS: on the proximal end of the gap (a), split by the break (b), and on the distal end of the gap (c).*

G-banding pattern typical for chromosomal segments harboring gene amplification [17].

Because SMAD5 and FRAC5 colocalize at region 5q31 and, a in few cases, the initiation of gene amplification in cancer cells was shown to occur at FS, chromosomes derived from normal lymphocytes cultures exposed to apc to induce the expression of FS were hybridized with the SMAD5 probe. Although FRA5C is not among the most frequently expressed FS, a sufficient number (25) of metaphases expressing FRA5C and showing informative signals were examined. The fluorescent signal for SMAD5 localized either centromerically or telomerically, or was divided by the gap of FRA5C in 17, 6, and 2 metaphases, respectively (Figure 3, *a–c*).

Southern blotting was performed to confirm that the *SMAD5* gene was amplified in Hep-40 cells. As a control for chromosome 5 copy number, the blot was also hybridized to a probe for the occludin gene, located at 5q12, outside of the amplified region. The relative intensity of the 8-kb *SMAD5 Hin*dIII band was considerably greater in DNA from the Hep-40 cell line, confirming the high degree of amplification of the gene (Figure 4).

To see whether the *SMAD5* gene is altered in primary HCC, we examined 17 specimens. Because the genomic DNA available was not sufficient for Southern blotting, the primary tumors and matching normal liver tissues were screened by semiquantitative PCR. In one tumor, a 50% decrease in the intensity of the SMAD5 PCR product was observed and, in another, a 40% increase was seen compared to an internal control and normal DNA counterparts, whereas in 15 tumors, no changes were detected. Representative examples are shown in Figure 5.

To correlate the copy number of SMAD5 signals with the expression of the gene, the levels of SMAD5 mRNA in the HCC cell lines were examined by Northern blotting. The SMAD5 probe hybridized to a major band of ~ 7.5 kb and a minor band of ~ 4 kb (not shown), as reported by others [18]. The size of the larger band is consistent with the

presence of a 5.2-kb 3′-untranslated region in the transcript (GenBank BK001394). SMAD5 mRNA was detected in all of the HCC cell lines and, in most cases, the levels were roughly similar to that found in normal liver, when normalized to the amount of ribosomal protein L19 mRNA (Figure 6). However, the SMAD5 mRNA level was 2.5-fold higher in the Hep-40 line that showed amplification of the gene, indicating that the increased gene dosage was associated with overexpression of the gene.

Discussion

In this analysis, we report genomic changes involving a region of chromosome 5 and changes in the *SMAD5* gene expression, which may be both relevant in the pathogenesis of human HCC. We demonstrated deletions and unbalanced
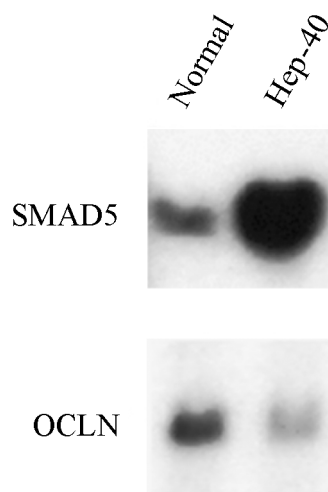

Figure 4. *Southern blot analysis of SMAD5 gene amplification. A Southern blot of HindIII-cut genomic DNA from normal human fibroblasts and the Hep-40 HCC cell line was hybridized to the SMAD5 cDNA probe (top) and the occludin (OCLN) probe (bottom). The SMAD5 and OCLN probes hybridized to single HindIII bands of 8 and 1.7 kb, respectively.*
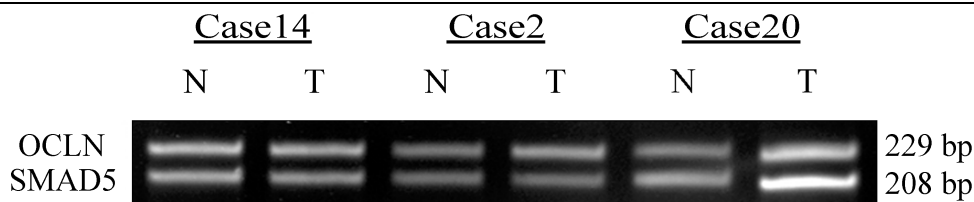
Figure 5. *Semiquantitative PCR analysis of SMAD5 gene in primary tumors. DNA from two primary HCC, Cases 2 and 20, displayed a 50% decrease and a 40% increase in the SMAD5 PCR products, respectively, compared to an internal control and normal DNA counterparts. Case 14 was similar to the remaining tumors, showing no difference between SMAD5 and OCLN.*

translocations with breakpoints near SMAD5 locus, the recurrent formation of isochromosome 5q leading to selective loss and gain of 5p and 5q, and the intrachromosomal amplification of *SMAD5* gene at the FS FRA5C indicating that this locus is a frequent target in HCC. However, CGH analyses performed by us and others have shown that chromosomal regions carrying other Smad genes (*SMAD1*, *SMAD2*, *SMAD4*, *SMAD7*, and *SMAD9*) are underrepresented in the majority of the cell lines examined here [12].

Over the last several years, the most frequently expressed, common apc-sensitive FS have been cloned and found to be the sites of deletion and amplification of cancer-related genes, as well as of integration of oncogenic viruses. Deletions of two tumor-suppressor genes located at common FS, FHIT at FRA3B and WWOX at FRA16D, have been observed in several types of cancer as well as numerous integrations of human papilloma viruses at FS in cervical carcinoma (reviewed in Refs. [19–23]).

Gene amplification is a common alteration in cancer cells, and like other structural changes, it is associated with genomic instability and frequently involves oncogenes whose increased expression confers a selective advantage on tumor cell growth [1,24]. Thus far, a few cases with a relatively low level of gene amplification mediated by breakage–fusion–bridge (BFB) cycles at FS have been demonstrated—the *MET* oncogene in gastric cancer through FRA7G and the *RIN* gene in oral cancer through FRA11B [25,26]. More recently,

DNA amplification through BFB cycles initiated by spontaneous telomere loss was shown in a human tumor cell line having a herpes virus thymidine kinase selectable marker integrated adjacent to the telomere on a marker chromosome 16 [27]. Chromosomal FS are regions prone to breakage, instability, and recombination [19–23]. FRA5C, although rather infrequently expressed, is no exception [28].

The marker chromosome in Hep-40 cells that carries multiple copies of SMAD5 is a complex rearrangement involving several chromosomes. Similar large markers were also observed in human tumor cells, and in rodent cells with amplification of drug resistance genes [25–27,29]. Such markers, which typically exhibit an abnormal banding pattern or homogeneously staining regions, are often apparent in tumor cells and may contain two or more amplified genes, frequently oncogenes. In a well-known breast carcinoma cell line SK-BR-3, we identified a marker originating from four chromosomes and containing multiple copies of the *ERBB-2* and *MYC* oncogenes on both chromosome arms [30]. The abnormal chromosome carrying the SMAD5 amplicon in Hep-40 tumor cells originated from five different chromosomes. The significant level of SMAD5 amplification in Hep-40 cells makes it difficult to determine how the amplification process was initiated. However, FISH analysis clearly showed that SMAD5 is located within FRA5C.

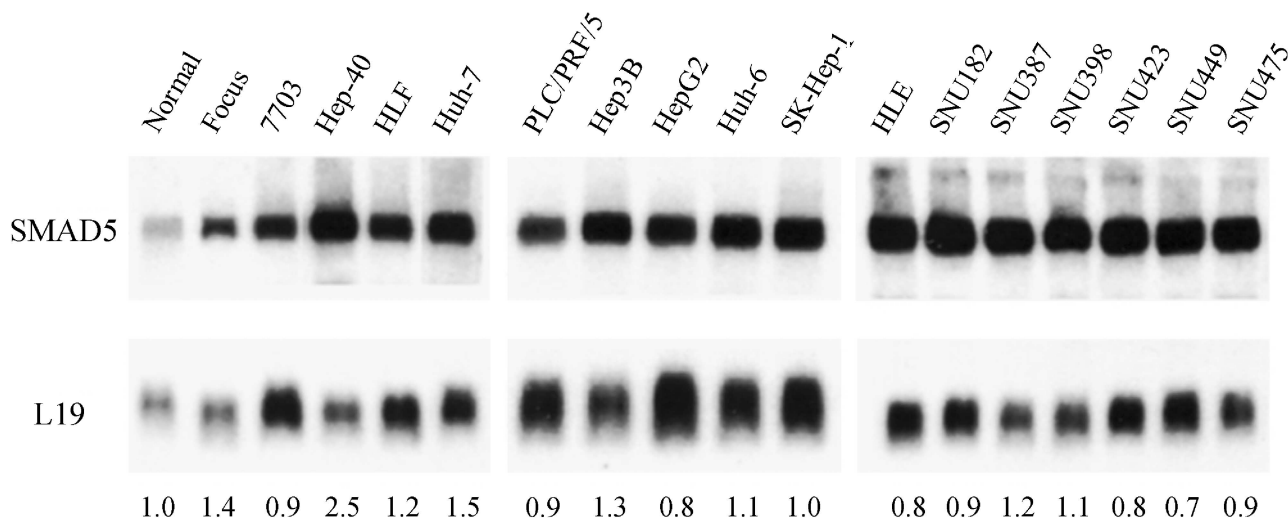This is consistent with other reports showing that FS could initiate gene amplification through BFB cycles [25–27,29].

Figure 6. *SMAD5 expression in HCC cell lines. Northern blots of total RNA from normal liver tissues and HCC cell lines were hybridized to the SMAD5 cDNA probe, and the major SMAD5 transcript of 7.5 kb is shown. As a control for loading, the blots were rehybridized to a mouse ribosomal protein L19 probe. The relative SMAD5 mRNA levels for each sample were calculated as the ratio of the intensities of the SMAD5 and L19 bands, divided by the ratio in normal liver cells.*

The complexity of the chromosome marker carrying the SMAD5 amplicon suggests breakage and interchromosome exchanges at two other FS on chromosome 5q and at non-FS regions. Recently, coamplification of the *MET* oncogene with three candidate tumor-suppressor genes was demonstrated in the GTL-16 gastric cancer cell line [31]. The size of the 5q amplicon in Hep-40 cells, however, has not been determined, and whether other genes are coamplified with SMAD5 is not known. No obvious candidate protooncogenes that could drive amplification map to the 5q31 region.

Our results *strongly* suggest that SMAD5 does not function as a tumor suppressor in HCC, as its expression is either maintained or upregulated by an increase in gene copy number or another mechanism. Localization of SMAD5 to a region at 5q31 commonly deleted in myeloid tumors indicated that it might be a tumor-suppressor gene, but no inactivating mutations have been found in the *SMAD5* gene when a number of different human primary tumors and cancer cell lines were analyzed [18,32,33]. In addition, increased expression of Smad5 was observed in colorectal cancer by immunohistochemistry [34,35]. Smad5 is activated by members of the bone morphogenetic protein (BMP) branch of the TGF-β superfamily [9]. The role of BMPs in the growth and differentiation of normal liver cells and in hepatocarcinogenesis has not been extensively studied. The HBV pX oncoprotein was found to enhance TGF-β and BMP-2 signaling by interacting with Smad4 [36]. Whether HBV infection affects the activity of Smad5 remains to be determined. Further investigations are needed to determine how alterations in the levels of Smad5 protein and its binding partners contribute to the process of hepatocarcinogenesis.

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