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Hisaki Nagai · Tetsuji Naka · Yoshie Terada
Toshiaki Komazaki · Aya Yabe · Enjing Jin
Oichi Kawanami · Tadamitsu Kishimoto · Noboru Konishi
Mitsutoshi Nakamura · Yasuhito Kobayashi · Mitsuru Emi

Hypermethylation associated with inactivation of the *SOCS-1* gene, a JAK/STAT inhibitor, in human hepatoblastomas

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Abstract We recently demonstrated inactivation in hepatocellular carcinomas (HCCs) of the gene encoding SOCS1/JAB1/SSI-1, a JAK-binding protein that regulates the JAK/STAT signal-transduction pathway. In a follow-up immunochemical investigation of expression of SOCS-1 in hepatoblastomas (HBLs), the protein was markedly reduced in half of the HBL tumors we examined. CpG-rich regions upstream of the *SOCS-1* gene were hypermethylated in 7 of the 15 HBL cases. The results suggest that hypermethylation may play an important role in silencing the *SOCS-1* gene, not only in adult HCCs, but also in liver tumors arising in childhood.

Key words Hepatoblastoma · SSI-1/SOCS-1/JAB · Methylation · JAK/STAT · Hepatocellular carcinoma

Introduction

Hepatoblastoma (HBL) is an embryonic tumor observed predominantly in children. However, the molecular mechanisms underlying transformation of liver cells in this condition remain obscure (for review, see Mascarello and Krous 1992). Cytogenetic abnormalities have been reported in a few cases of HBL, among them trisomy of chromosomes 20, 2, and 8, and of the long arm of chromosome 1 (Mascarello and Krous 1992; Tonk et al. 1994; Fletcher et al. 1991; Bardi et al. 1992; Simon et al. 1991; Dressler et al. 1993), although these features are not observed in a majority of karyotyped HBL tumors. However, loss of heterozygosity (LOH) around 11p15.5 is a frequent observation in HBLs (Simms et al. 1995), and abnormalities in that region are associated with Beckwith-Wiedeman syndrome (BWS). Recently, Hatada et al. (1996) showed that the $p57^{Kip2}$ gene is imprinted in BWS; alteration of this gene might be involved in the development of HBLs as well. Other investigators have described a mutation of the *CTNNB1* gene in HBLs that results in intense cytoplasmic staining of beta-catenin and accumulation of this protein in the nuclei of tumor cells, but that observation has not been confirmed by other laboratories (Blaker et al. 1999). In our own recent deletion-mapping study, we defined an interstitial deletion at 4q21–22 in the germline of an HBL patient within an interval of 8 cM between D4S2964 and D4S2966, which overlaps with the commonly deleted region previously described in adult hepatocellular carcinomas (HCCs; Terada et al. 2001). Those findings suggest that certain genetic events may occur in both types of hepatic malignancy.

We and others previously cloned the *SOCS1* gene (suppressor of cytokine signaling 1, also termed JAB1 and SSI-1), encoding a JAK-binding protein that regulates the signal-transduction pathway of JAK/STAT (Janus kinase signal transducers and activators of transcription). SOCS1 protein relays signals into cells from various cytokines in the extracellular matrix (Naka et al. 1997; Starr et al. 1997; Endo et al. 1997). We and others have described inactivation by methylation of the *SOCS1* gene in HCCs (Nagai et al. 2001; Yoshikawa et al. 2001).

In the present study, we investigated 15 human HBL tumors for SOCS1 abnormalities, and identified in a substantial portion of HBL tumor tissues a marked reduction of SOCS1 expression and associated hypermethylation of the gene's GC-rich region. The results suggest that dysregulation of the JAK/STAT signal transduction path-

H. Nagai · Y. Terada · T. Komazaki · A. Yabe · E. Jin · O. Kawanami · M. Emi (✉)
Department of Molecular Biology and Pathology, Institute of Gerontology, Nippon Medical School, 1-396 Kosugi-cho, Nakahara-ku, Kawasaki 211-0063, Japan
Tel. +81-44-733-5230; Fax +81-44-733-5192
e-mail: memi@nms.ac.jp

T. Naka · T. Kishimoto
Department of Medicine III, Osaka University Medical School, Osaka, Japan

N. Konishi · M. Nakamura
Department of Pathology, Nara Medical University, Kashihara, Japan

Y. Kobayashi
Department of Pathology, Saitama Cancer Center Hospital, Saitama, Japan

way plays a role in the development of this embryonic liver tumor.

Materials and methods

Tumor specimens and DNA extraction

Tumor samples and corresponding noncancerous tissues were obtained from 15 patients with HBL who underwent surgery at Saitama Cancer Center Hospital. The study was approved by the Institutional Review Board, and informed consent was obtained from the families of all participants prior to surgery. Clinical and pathology data are listed Table 1. Portions of these tissues were frozen immediately after surgery and stored at −80°C. DNAs were extracted later according to standard procedures.

Immunohistochemistry of SOCS1 protein in HBL tumors

Immunohistochemical staining was performed with mouse anti-SOCS1 polyclonal antibody on formalin-fixed, paraffin-embedded HBL tissues using the streptoavidin biotin peroxidase complex method. After heat treatment, peroxidase activity was suppressed by 3% hydrogen peroxide in methanol for 30 min. Primary antibody was applied to the slides and incubated overnight at 4°C. SOCS1 protein was detected by SAB-Po kit (Nichiren Corporation, Tokyo, Japan). The sections were treated with biotinylated rabbit anti-mouse immunoglobulins (Dako code # E0432) for 1 h. After three washes with phosphate-buffered solution (PBS), samples were incubated with 100 μg/ml streptoavidin (Dako code #K0377A) and biotinylated horseradish peroxidase (Dako code #K0377B) for 10 min, followed by three more washes in PBS. The slides were covered with 0.05% 3-3′ diaminobenzidine containing 0.01% hydrogen peroxide for 7 min, rinsed with distilled water, dehydrated in ethanol, and overlaid with coverglasses using mounting medium.

Methylation assay by bisulfite-modified polymerase chain reaction (PCR)

One microgram of each DNA sample in a volume of 50 μl was denatured by NaOH (final concentration, 0.2 M) for 10 min at 37°C. For samples containing only nanogram quantities of human DNA, 1 μg of salmon-sperm DNA (Sigma, St. Louis, MO, USA) was added as a carrier before modification. Thirty microliters of 10 mM hydroquinone (Sigma) and 520 μl of 3 M sodium bisulfite (Sigma) at pH 5, both freshly prepared, were added and mixed, and the samples were incubated under mineral oil at 50°C for 16 h. Bisulfite-modified DNAs were purified using the Wizard DNA purification resin according to the manufacturer's instructions (Promega, Madison, WI, USA) and eluted into 50 μl of water. Modification was completed by treatment with NaOH (final concentration, 0.3 M) for 5 min at room temperature, followed by ethanol precipitation. Each DNA sample was resuspended in water and either used immediately or stored at 22°C.

Aliquots of unmodified DNA (50–100 ng) in final volumes of 50 μl included 5% dimethyl sulfoxide. All DNAs were amplified using primers derived from the nucleotide sequence of the *SOCS1* gene deposited in the Genome Database (GDB) (accession number U88326). Primers were designed to amplify the GC-rich region within the *SOCS1* promoter, as follows: 5′-GTTGTAGGATGGGG TCGCGGTCGC-3′ and 5′-CTACTAACCAAACTAAA ATCCGCG-3′ for detection of methylated alleles, and 5′-GTTGTAGGATGGGGTTGTGGTTGT-3′ and 5′-CTAC TAACCAAACTAAAATCCACA-3′ for detection of unmethylated alleles.

Analysis of the genomic SOCS1 region in HBLs

Genomic DNAs from the 15 HBL tumors were screened for mutations using a single pair of primers for the PCR (5′GGAAGAGCTCCGCGGAGTACAGAGCCCATT-3′ and 5′-AACCCAGGCCGGGGAGGGTACCCACAG GT-3′) that were designed to amplify the entire coding region of the *SOCS1* gene. The PCR products were sequenced directly using primers designed to sequence overlapping segments. Nucleotides were determined by the BigDye method terminator cycle-sequencing method (Applied Biosystems, Foster City, CA, USA) using an autosequencer (ABI PRISM 377; Applied Biosystems).

To examine allelic losses at 16p13.13, we used the chromosomal location of the *SOCS1* gene, primers GT356F (5′-TAGGTACAGTGACCTAAAGC-3′) and GT356R (5′-CTGCTGAATCATGAAGCTGA-3′), to amplify the dinucleotide-repeat sequence present on a genomic cosmid clone, super-cos 356d7 (GDB #AC002286), which contains both the CA-repeat and the entire *SOCS1* gene (Naka et al. 1997).

Results

Reduced staining of SOCS1 in HBL tumors

Figure 1 displays representative immunohistochemical staining of an HBL tumor section with anti-SOCS1 antibody. Seven of the 15 HBL specimens showed absence or marked reduction of SOCS1 protein in the cytoplasm.

Bisulfite-modified amplification of the *SOCS1* promoter region

Previously we and others had demonstrated hypermethylation and combined loss of the remaining allele as the predominant mechanisms for silencing the *SOCS1* gene in human HCCs (Naka et al. 1997; Starr et al. 1997). To investigate the methylation status of the *SOCS1* promoter region in HBLs, we performed PCR experiments based on bisulfite modification of DNA from all 15 HBL tumor specimens.

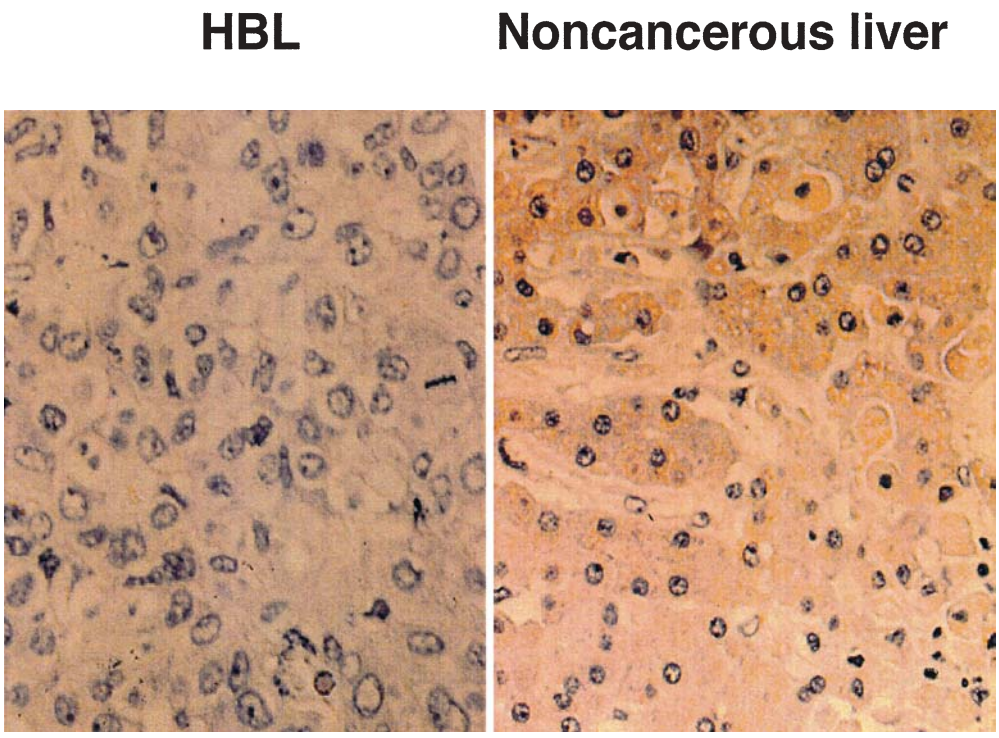

Fig. 1. Representative photomicrographs illustrating immunohistochemical staining of SOCS1 protein in the cytoplasm of normal hepatocytes and marked reduction in hepatoblastoma (*HBL*) cells

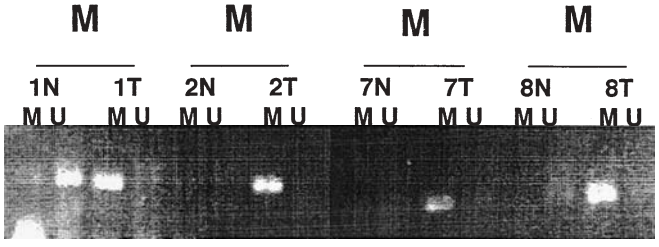

Fig. 2. Bisulfite-modified polymerase chain reaction (PCR) assay of methylation status of the *SOCS1* gene in eight primary HBL tumors and paired nontumorous tissues. For the modification experiments, we used 1-μg aliquots of DNA from HBLs (*T*) and adjacent nontumorous liver cells (*N*). PCR amplifications involved methylation-specific primer pairs for detection of methylated alleles (*M*) and unmethylation-specific primer pairs for unmethylated alleles (*U*). The majority of the tumor samples shown here were hypermethylated

Figure 2 displays representative results, which demonstrate that, in multiple cases, HBL tumor-derived DNA revealed no unmethylated allele(s). We observed hypermethylation of *SOCS1* alleles in 7 of the 15 HBLs examined (tumors T1, T2, T7, T8, T9, T10, and T11; summarized in Table 1). Six of the hypermethylated tumors had shown marked reduction of SOCS1 expression, as revealed by immunohistochemistry (Table 1).

Hypermethylation as a mechanism for inactivating *SOCS1*

We investigated the possibility of other genetic mechanisms to account for inactivation of the *SOCS1* gene in the HBL tumors, specifically allelic loss at 16p13.13 and somatic mutation within the *SOCS1* gene. Analysis of LOH using a microsatellite DNA marker (GTcos356) that lies approximately 3 kb downstream of the translation–initiation site of *SOCS1* within the genomic cosmid clone 356d7, demonstrated retention of heterozygosity at this locus for all 13 HBL tumors that were informative for the marker. Moreover, direct sequencing of the entire coding region revealed no somatic mutations in the *SOCS1* gene in any of the 15 HBL tumors. These results, taken together with the results of our methylation analysis, suggest that hypermethylation of the promoter region is the predominant mechanism for inactivation of the *SOCS1* gene in HBL tumors.

Discussion

Most embryonic tumors may be defined as immature tissue that continues to proliferate inappropriately because of a disturbance of the growth mechanism at an early stage of differentiation (Koufos et al. 1985). That notion is supported by evidence showing blocked ontogeny/maturation arrest of stem cells, in which aberrant differentiation and proliferation of stem cells eventually induce malignant transformation. HBL may belong to a class of tumors that arise through genetic alteration at the stem-cell level, although a number of genetic alterations may underlie the genesis of HBL. For example, the association of HBL tumors with an impaired imprinted gene in BWS patients, recently described, suggests that epigenetic mechanisms such as methylation status play roles in HBL development (Sell 1993).

Hypermethylation is recognized as one of the mechanisms that can inactivate tumor suppressor genes, in this

Table 1. Clinical and pathology data and summary of genetic and epigenetic changes of *SOCS1* in HBL

No.	Age	Sex	Pathology	Karyotype	Methylation of *SOCS1*[a]	Immunohistochemistry[b]
1	6 m	F	Embryonal	46,XX	+	−
2	5 m	F	Fetal	46,XX	+	−
3	1 y	F	Fetal	46,XX	−	+
4	1 y	M	Poorly defined/mesenchymal	4q+	−	+
5	6 m	M	Well defined	46,XY	−	−
6	8 m	M	Well defined	46,XY	−	+
7	8 y	M	Macrotrabecular	46,XY	+	+
8	13 y	M	Well defined	ND	+	−
9	4 m	M	Embryonal	48,XY,+8+20	+	−
10	11 m	M	Fetal	46,XY	+	−
11	1 m	M	Well defined	ND	+	−
12	2 y	M	Embryonal	48,XX,+2,+5,+8,−22	−	+
13	11 m	M	Well defined	46,XY	−	+
14	3 y	F	No data	ND	−	+
15	5 y	F	Mixed (fetal, embryonal)	ND	−	+
16	82 y	M	Poorly defined/mesenchymal	ND	ND	ND

HBL, Hepatoblastoma; m, months; y, years; F, female; M, male; ND, not determined
[a] +/− indicate methylation/unmethylation of the *SOCS1* gene
[b] Immunohistochemistry indicates the positive (+) or negative (−) signals in HBL

case by repressing their transcription (Jones and Laird 1999). An increasing number of examples include hypermethylation of *RB1*, *VHL*, *MLH1*, *CDKN2A*, *CDKN2B*, *APC*, or *H-cadherin* in various types of cancers (Sato et al. 1998). We have documented here a reduction in *SOCS1* expression in HBLs when this gene or its promoter region is hypermethylated. Hypermethylation of *SOCS1* was in fact a frequent event in the HBLs we examined, indicating that this mechanism plays a predominant role in the oncogenesis of hepatic precursor cells. The chromosomal region harboring the *SOCS1* gene, 16p13-pter, is a known hotspot of hypermethylation in HCCs as well (Kanai et al. 1996); moreover, the region commonly deleted in HCCs in our previous study overlaps the *SOCS1* locus. The discrepancy between HCC and HBL as regards allelic loss around the *SOCS1* locus underscores the point that different epigenetic mechanisms may operate in the two types of hepatic tumor, especially in relation to the genomic imprinting recently described in this chromosomal region.

SOCS1 functions as a negative regulator of the JAK/STAT signaling pathway (Wen et al. 1995). Emerging evidence strongly implicates abnormal activation of JAK/STAT signaling during oncogenic transformation. Constitutive activation of Stat3 proteins is frequent in tumor cells (Wen et al. 1995), suggesting that aberrant Stat3 signaling plays a part in malignant progression. The STAT proteins are latent transcriptional factors that are activated by phosphorylation of tyrosine residues in response to interaction with cytokine receptors at the cell surface. In regenerating liver, interleukin-6 may induce hepatocyte proliferation by activating STAT3 (Ichiba et al. 1998; Narazaki et al. 1998). Therefore, dysregulation of this pathway might profoundly alter growth of liver cells (Blaker et al. 1999). In the present study, we demonstrated that the normal suppression of the JAK/STAT signaling pathway by SOCS1 is impaired in HBL tumors by an epigenetic mechanism. Identification of the precise mechanism(s) controlling differentiation of hepatocytes from hepatic stem cells will help to clarify the pattern of oncogenesis of HBL.

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