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Expression of the Musashi1 gene encoding the RNA-binding protein in human hepatoma cell lines☆

Hong-Jin Shu,[a] Takafumi Saito,[a,*] Hisayoshi Watanabe,[a] Jun-Itu Ito,[a] Hiroaki Takeda,[a] Hideyuki Okano,[b] and Sumio Kawata[a]

[a] *Second Department of Internal Medicine, Yamagata University School of Medicine, Yamagata 990-9585, Japan*
[b] *Department of Physiology, Keio University School of Medicine, Tokyo, Japan*

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Abstract

Musashi1, a neural RNA-binding protein, plays an important role in regulating cell differentiation in precursor cells. Recently, expression of Musashi1 has been detected in human tumor tissues such as gliomas and melanomas, suggesting its involvement in oncogenic development. To determine any association between Musashi1 and the development of liver cancer, we investigated its gene expression in seven human hepatoma cell lines: HuH6, HuH7, Hep3B, SK-Hep1, HepG2, HLE, and HLF. Musashi1 mRNA expression was analyzed using the reverse-transcription polymerase chain reaction (PCR), and the PCR products were sequenced using a subcloning procedure. Musashi1 protein expression was analyzed in HuH7 and HepG2 cells by Western blot and immunofluorescence staining. Musashi1 mRNA was detected in the HuH6, HuH7, and Hep3B hepatoma cell lines, but not in the others. Sequencing of the PCR-amplified Musashi1 cDNA in these three cell lines showed the expected sequence of the human Musashi1 gene. Musashi1 protein expression was confirmed in HuH7 cells, which were positive for Musashi1 mRNA expression, but not in HepG2 cells. These results suggest that Musashi1 expression may be an important factor in the development of several types of carcinoma such as human hepatoma, and may be a useful molecular marker for tumor detection.

Keywords: Musashi; RNA-binding protein; Liver; Hepatoma; Carcinogenesis

Musashi1, a neural RNA-binding protein, was first isolated as a mammalian homolog of the *Drosophila* protein, which is required for the asymmetric division of sensory neural precursor cells [1]. In mice, Musashi1 has been detected in post-natal neural precursor cells, which are closely involved in the asymmetric division that generates differentiated cells, as well as in neural precursor cells during the embryonic development of the central nervous system (CNS) [2,3]. In humans, Musashi1 is also expressed in CNS stem cells and neural progenitor cells with a close relationship to cell differentiation, and can be regarded as an evolutionarily conserved marker for them [4–6]. Musashi1 expression is down-regulated as cell differentiation progresses, and expression persists into adulthood only at very low-levels and in a few organs, such as the brain, testis, and intestine [3]. Musashi1 is also known to regulate positively the Notch signaling pathway [6,7] thereby maintaining the self-renewing ability of stem cells [8]. Furthermore, since the gene-expression patterns of tumor cells may resemble those of their precursor cells, and recent investigations on CNS tumors have shown that Musashi1 is expressed in human gliomas [9,10], suggest that the potential significance of Musashi1 expression in other tumor cells would be worthwhile.

Liver cells are classified into two types: hepatocytes and cholangiocytes. Both are considered to originate from liver stem cells [11–14]. Although it remains unknown whether Musashi1 is expressed after liver injury in adults, it is not detected in normal, adult human liver

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* Corresponding author. Fax: +81-23-628-5311.
E-mail address: tasaitoh@med.id.yamagata-u.ac.jp (T. Saito).

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tissue by reverse-transcription polymerase chain reaction (RT-PCR) analysis [9]. The most common histological types of primary liver cancer are hepatoblastoma and hepatocellular carcinomas, and in both types the tumor cells retain features of liver stem cell differentiation [15]. To date, the expression of Musashi1 in carcinoma tissues such as liver cancer cells has not been investigated. However, if present, it may provide an important new marker for the diagnosis of human hepatomas.

In this study, we investigated the expression of the Musashi1 gene in various human hepatoma cell lines using RT-PCR analysis, and found that several cell lines were positive. This is the first report of Musashi1 gene expression in human hepatoma cells.

Materials and methods

Cell lines and culture. Seven human hepatoma cell lines, HuH6, HuH7, Hep3B, SK-Hep1, HLE, HLF, and HepG2, were used for the mRNA gene-expression analysis of Musashi1. The cells were seeded into 250 ml flasks and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 units/ml streptomycin, and 10% fetal bovine serum at 37 °C in a humidified atmosphere containing 5% CO_2. All media and chemicals used in the cell cultures were obtained from GIBCO BRL (Gaithersburg, MD).

RNA isolation. Cells were harvested with 0.25% trypsin and suspended in complete DMEM. Their total cellular RNAs were then extracted using the Isogen kit (Wako Pure Chemical Industries, Osaka, Japan) according to manufacturer's instructions. The RNA concentrations were determined by ultraviolet spectrophotometry (UV-1200 spectrophotometer, Shimazu, Kyoto, Japan).

RT-PCR, cloning, and nucleotide sequencing. Complementary DNA (cDNA) was synthesized by incubating the total RNA template (3 μg) and 200 units Moloney murine leukemia virus reverse transcriptase with 50 pmol random primers (GIBCO BRL) in a total reaction volume of 20 μl. The cDNA (1 μl) was then amplified by PCR using specific primers based on published nucleotide sequences of cloned human brain Musashi1 [4]. The nucleotide positions (nt) were numbered according to these published sequences. Primers were then designed to produce a 524-bp amplification product (nt 254–795). These were: 5′-GGC TTC GTC ACT TTC ATG GAC CAG GCG-3′ and 5′-GGG AAC TGG TAG GTG TAA C-3′. The PCR consisted of 40 cycles at a denaturation temperature of 94 °C for 30 s, an annealing temperature of 58 °C for 2 min, and an extension temperature of 72 °C for 1 min. Molecular cloning was performed with an Original TA cloning kit (Invitrogen, Carlsbad, CA). The PCR product was ligated into the pCR 2.1 vector and transfected into competent cells. Plasmid DNA was then amplified in *Escherichia coli* and purified using a Qiagen purification kit (Qiagen, Chatsworth, CA). The insert DNA was sequenced using the 5′ sequencing primer (M13 reverse primer) 5′-CAG GAA ACA GCT ATG AC-3′ and the 3′ sequencing primer (M13 forward primer) 5′-GTA AAA CGA CGG CCA G-3′ by the fluorescent dye terminator cycle method in an ABI 310 automated sequencer (Applied Biosystems, Foster City, CA). The nucleotide sequences were then aligned with the reported sequences of human Musashi1. The PCR for β-actin was performed as an internal control (551 bp) [9].

Western blot analysis. The proteins for Western blot analysis were prepared by the treatment of HuH7 and HepG2 cells with cell lysis buffer (25 mM Tris–HCl, pH 7.4, 50 mM NaCl, 0.5% Na-deoxycholate, 100 mM NaF, 2% NP-40, 0.2% SDS, 200 μM $NaVO_3$, 0.57 mM phenylmethylsulfonyl fluoride, 1 M benzamidine, 10 μg/ml leupeptin, 2 μg/ml pepstatin A, and 1 μg/ml aprotinin), followed by centrifugation at 15,000 rpm at 4 °C for 20 min. The supernatant was stored at −70 °C. The protein concentrations within each homogenate were determined with the Pierce Coomassie protein assay reagent (Pierce Chemical, Rockford, IL). A 15-μg sample of proteins was subjected to a 12.5% SDS–polyacrylamide ready gel (Bio-Rad Laboratories, Richmond, CA). Resolved proteins were electrophoretically transferred to an Immunobilon-P membrane (Millipore, Bedford, MA) at 4 °C and processed for immunodetection. After blocking with 5% nonfat milk for 1 h at room temperature, the membrane was incubated with specific rat monoclonal antibodies recognizing the Musashi1 protein (anti-Musashi1) [5] at 1:500 dilution at 37 °C for 2.5 h. The membrane was then incubated with alkaline phosphatase-conjugated goat anti-rat IgG antibody (Cappel, Aurora, OH) at 1:1,000 dilution for 1.5 h at room temperature. Detection was performed with the BCIP/NBT phosphate substrate system, according to manufacturer's protocol (KPL, Gaithersburg, MD).

Immunofluorescence and immunoperoxidase staining. Musashi1 protein was analyzed by indirect immunofluorescence staining in cells. Expression of alpha-1-fetoprotein (AFP) and cytokeratins (CKs), which act as hepatocyte and biliary cell markers, respectively, was also analyzed using polyclonal goat anti-human AFP and monoclonal mouse anti-human CK18 and CK19 (DAKO, Kyoto, Japan). Fluorescein isothiocynate-labeled $F(ab')_2$ fragments of immunoglobulins against the species of origin of the primary antibodies (DAKO Japan, Kyoto, Japan) were used as secondary antibodies. The cells were washed in phosphate-buffered saline, pH 7.2 (PBS), placed onto a 10-well slide, air-dried, and fixed in cold acetone for 5 min. They were then incubated with the primary antibodies at 37 °C for 1 h, washed three times in PBS, incubated with the secondary antibodies at 37 °C for 1 h, washed three times in PBS, and examined under a fluorescence microscope (Olympus BX-FLA; Olympus Optical, Tokyo, Japan).

Musashi1 protein was also examined by immunoperoxidase staining in fetal mouse liver. Fetal mouse liver (14.5 gestational days) specimens were fixed with 10% formalin and embedded in paraffin. The sections (4 μm) were prepared, deparaffinized, and washed extensively with PBS, before immunostaining. The Musashi1 protein was unmasked by microwaving at 800 W for 20 min in 0.01 M citrate buffer, pH 6.0. An avidin–biotin complex immunoperoxidase staining technique was employed (Vectastain ABC kit; Vector Laboratories, Burlingame, CA). After inhibiting the endogenous peroxidase activity of the liver tissue with 0.3% hydrogen peroxidase solution for 30 min at 4 °C, the sections were incubated with anti-Musashi1 at 1:200 dilution in a moist chamber at 4 °C overnight, followed by incubation with the biotinylated secondary antibodies and the avidin–biotin complex according to manufacturer's instructions. The reaction products were stained with a 0.02% solution of 3, 3′-diaminobenzidine in 0.05 M Tris–HCl buffer, pH 7.2, containing 0.01% hydrogen peroxide. The slide was counterstained with hematoxylin, dehydrated, and mounted. As a negative control PBS was used instead of primary antibody in both immunofluorescence and immunoperoxidase staining.

Results

Musashi1 protein expression in fetal mouse liver

In mouse fetal liver at 14.5 gestational days, the Musashi1 protein was detected in the cytoplasm of early hepatocytes (Fig. 1).

Musashi1 mRNA expression in human hepatoma cell lines

The PCR-amplified product obtained using the Musashi1-specific primers produced clear bands of the

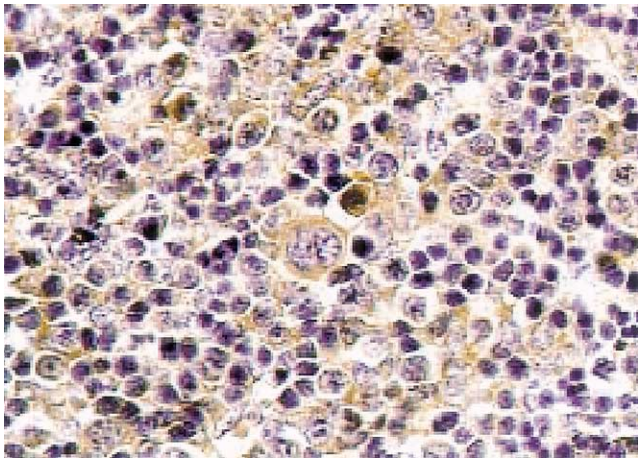

Fig. 1. Immunohistochemical study of Musashi1 protein expression in fetal mouse liver (14.5 gestational days). Musashi1 protein was detected in the cytoplasm of early hepatocytes. ×400.

predicted 542-bp size. Musashi1 gene expression was observed in three hepatoma cell lines (HuH6, HuH7, and Hep3B) but was not detected in the other four hepatoma cell lines (SK-Hep1, HepG2, HLE, and HLF) (Fig. 2). Sequencing of the PCR-amplified Musashi1 cDNA in the three positive hepatoma cell lines showed the expected sequence of the human Musashi1 gene. Although one to three single point substitutions were found between nt 737 and 778 in these cell lines, they were not detected in cell genomic DNA. The normal rat liver tissue used as a control was negative for Musashi1 gene expression, when we examined by RT-PCR using the specific primers for Musashi1 that are identical in nucleotide sequences between human and rat.

Musashi1 protein expression in HuH7 and HepG2 cell lines

Western blot analysis and immunofluorescence staining for Musashi1 protein were performed in HuH7 cells (which were positive for Musashi1 mRNA expression) and HepG2 cells (which were negative for Musashi1 mRNA expression). Western blot analysis showed

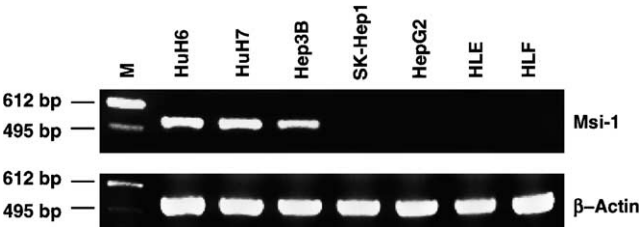

Fig. 2. RT-PCR analysis of Musashi1 mRNA expression in human hepatoma cell lines. Musashi1 mRNA was detected in the HuH6, HuH7, and Hep3B cell lines. No Musashi1 gene expression was observed in the SK-Hep1, HepG2, HLE, and HLF cell lines. The predicted size of the PCR-amplified Musashi1 product was 542 bp.

that the Musashi1 protein was detected with a molecular mass of approximately 40 kDa in HuH7 cells but it was not detected in HepG2 cells (Fig. 3). Musashi1 protein expression was confirmed in the cytoplasm of all the HuH7 cells by immunofluorescence staining (Fig. 4), while the HepG2 cells were negative for Musashi1 protein. We also tested for hepatocyte and biliary cell markers (AFP and CKs, respectively) in HuH7 cells and HepG2 cells. Both types of markers were expressed in these cells (Fig. 5).

Discussion

The Musashi1 gene, which encodes a neural RNA-binding protein, is closely associated with cell differentiation in CNS stem cells [1–5]. In the endodermal cells, Musashi1 is known to be expressed in intestinal epithelial stem cells [16]. We demonstrated in this study that, in the mouse, Musashi1 is expressed in early hepatocytes during the embryonic stage of liver development. Abnormalities of cell-regulatory gene expression may be involved in carcinogenesis; however, little is known about the relationship between Musashi1 gene

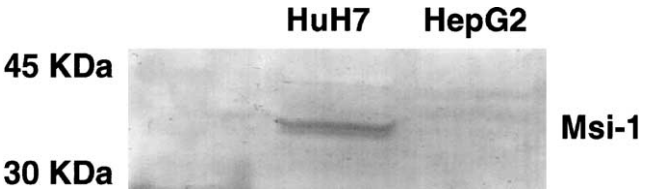

Fig. 3. Western blot analysis of Musashi1 protein expression in HuH7 and HepG2 cells. Musashi1 protein was detected with a molecular mass of approximately 40 kDa in HuH7 cells, but it was not detected in HepG2 cells.

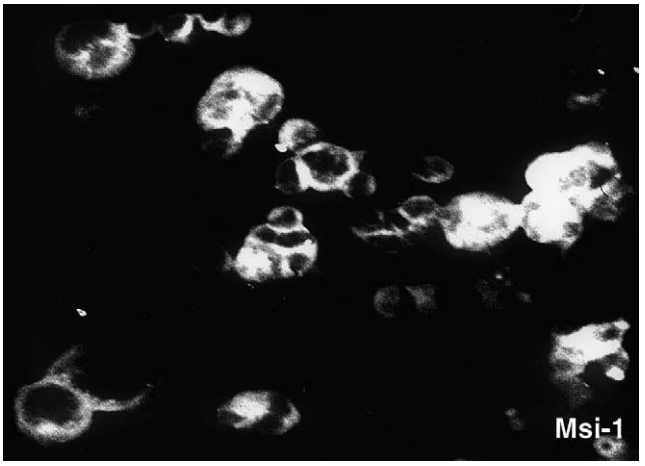

Fig. 4. Immunofluorescence staining for Musashi1 protein. Musashi1 protein was detected in the cytoplasm of all the HuH7 cells. ×400.

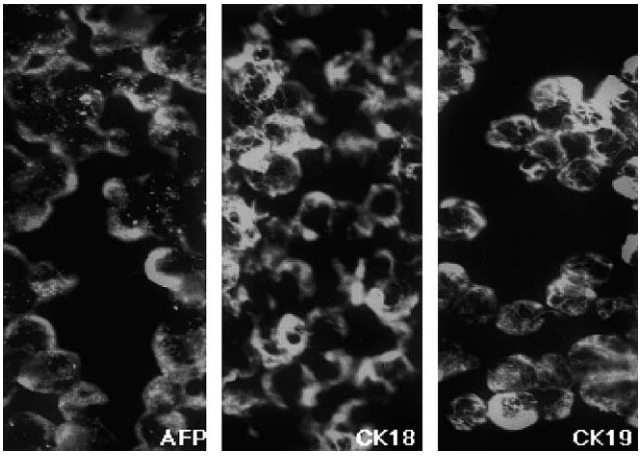

Fig. 5. Immunofluorescence staining for hepatocyte and biliary cell markers in HuH7 cells. Alpha-1-fetoprotein (AFP), cytokeratins (CK)18 and CK19 were observed in the cytoplasm of all the HuH7 cells. ×400.

expression and tumor development in the liver. In this study, we showed for the first time that the Musashi1 gene, which is not expressed in the normal adult human liver [9], is detectable in several human hepatoma cell lines, namely HuH6, HuH7, and Hep3B. Musashi1 protein expression was also confirmed in HuH7 cells.

The sequence of the PCR-amplified Musashi1 cDNA in the three positive hepatoma cell lines showed the expected sequence in frame, when compared with the reported sequence of the human Musashi1 gene [4]. Although one to three single point substitutions were found in their sequences, they were not detected in cell genomic DNA. This may be due to the RNA editing, the post-transcriptional alteration of a gene-encoded sequence, in eukaryotes [17,18]. The RNA editing may result in the production of functionally distinct proteins from a single gene. Even though this does not alter the predicted sequence of the protein, it is possible that some regulatory functions associated with this point substitution could alter the expression of the gene; a single base alteration can change the affinity of protein–DNA or protein–RNA binding, or the secondary structure of the mRNA, thus changing gene expression dramatically [19]. It remains unclear whether there is any relationship between these substitutions and cellular dysfunction. However, substitution of the Musashi1 gene may alter the expression of proteins important for the normal growth of hepatocytes, and such substitutions could be acquired by cancer cells. Further investigations are needed to elucidate the biologiocal significance of their nucleotide substitutions found in the Musashi1 cDNA.

Human hepatoma is a common disease that causes liver insufficiency. To investigate its cell origins can provide better understanding of its development and improve the strategy to approach of diagnosis. In this study, we showed that some hepatoma cell lines expressed Musashi1, the excellent marker for CNS stem cells. Thus it is possible that human hepatoma cells share some of the same origins and characteristics of stem cells. Functional research on Musashi1 has shown that Notch signaling activity is increased by Musashi1 expression, together with post-transcriptional down-regulation of the mammalian numb gene [7]. Activation of the Notch signaling pathway suppresses the differentiation of stem cells and plays a fundamental role in maintaining cells in an undifferentiated state. Signals exchanged between neighboring cells through the Notch receptor eventually dictate the fate of an individual cell [8,20], and inappropriate activation of the Notch signaling pathway may be involved in the development of neoplasms. The relationship between Musashi1 expression and the activation of Notch signaling in hepatoma cell lines is currently being investigated in our laboratory.

Recently, expression of the Musashi1 gene was observed in human gliomas, suggesting a relationship between CNS developmental processes and oncogenesis [9,10]. The extent of Musashi1 expression was also associated with the grade of malignancy of the glioma. In the present study, no relationship was observed between the grade of malignancy and the degree of Musashi1 expression in human hepatome cells, since Musashi1 was well expressed in both hepatoblastoma (HuH6) and well-differentiated hepatoma cell lines (HuH7 and Hep3B). Hepatoma cells which were positive for Musashi1 protein expression (HuH7) possessed both biliary cell and hepatocyte markers, indicating that they had retained features of the hepatic differentiation process. However, the HepG2 cells which were negative for Musashi1 protein expression also possessed such markers. Thus, further studies will be needed to clarify precisely how Musashi1 is associated with the oncogenic development in liver cells.

In conclusion, Musashi1 expression may be an important factor in the development of several human hepatoma cell lines, and may act as a useful molecular marker for tumor detection.

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