Human Homologue of Maid Is a Useful Marker Protein in Hepatocarcinogenesis

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Background & Aims: **Human homologue of maid (HHM) is a helix-loop-helix (HLH) transcriptional regulatory protein that is involved in the hepatic stem cell development and differentiation. We analyzed the potential involvement of HHM in hepatocarcinogenesis.** ***Methods:*** **We analyzed HHM expression in the choline-deficient L-amino acid defined (CDAA) diet model of rat hepatocarcinogenesis and in human adenomatous hyperplasia (AH) and hepatocellular carcinoma (HCC) biopsy samples. We assessed the effects of HHM on cell proliferation. We screened proteins that bind to HHM protein using a yeast 2-hybrid screen.** ***Results:*** **High HHM expression was seen in foci and HCC induced in the rat CDAA diet model. HHM protein was expressed in 23 of 32 AH samples (72%), 19 of 28 well-differentiated HCC samples (68%), and 9 of 18 poorly-moderately differentiated HCC samples (50%). Over-expressed HHM enhanced the S phase. HHM interference RNA significantly inhibited cell proliferation. A yeast 2-hybrid screen identified Jun activation domain-binding protein 1 (Jab1) as a binding partner for HHM. We confirmed HHM and Jab1 binding by immunoprecipitation and immunofluorescent histochemistry. The expression of Jab1 was found in human AH and HCC samples. We found an association between levels of expression of HHM and those of Jab1 in AH and HCC tissues examined ($P = .027$ by χ^2 test).** ***Conclusions:*** **High-level HHM expression was found from the very early stages of hepatocarcinogenesis, suggesting that HHM may be a useful marker protein to detect.**

Helix-loop-helix (HLH) transcriptional regulatory proteins regulate transcription during development and differentiation of various organs and are roughly divided into 7 groups with respect to tissue distribution, dimerization capabilities, and DNA-binding specificities.[1,2] Ubiquitously expressed class I HLH proteins, such as E12 and HEB, form heterodimers with tissue-specific class II HLH proteins, such as MyoD and BETA2/NeuroD. This heterodimer binds to specific DNA-binding sites, such as E-Box, in the promoter region of tissue-specific genes. In the development and differentiation of biliary epithelial cells, class VI HLH protein HES1 (hairy and enhancer of split 1) is important. Expression of HES1 is regulated by the Notch pathway,[3] and mutations in the Jagged 1 gene, which encodes a Notch ligand, induce Alagille syndrome (syndromic bile duct paucity).[4,5] We therefore hypothesized that there is a hepatocyte-specific HLH transcriptional regulatory protein in the liver and then attempted to clone HLH transcriptional regulatory proteins from a human fetal liver cDNA library using E12 protein as a bait protein.[6] Human homologue of maid (HHM) was identified in this manner, and, although it is not a class II HLH protein, it appears to be a human homologue of murine maternal Id-like molecule (Maid), which was believed to be a new class V dominant inhibitory HLH protein. Id family proteins are class V dominant inhibitory HLH proteins that regulate cell proliferation and differentiation by inhibition of class I and class II HLH protein dimerization. HHM is larger than Id family proteins, consisting of 360 amino acids, and has a leucine zipper motif. The HLH structure of HHM is different from that of Id. HHM specifically inhibited the luciferase gene activation induced by hepatocyte nuclear factor 4 (HNF4) promoter, but Id2 did not inhibit the luciferase gene activation.[6] The murine Maid molecule

Abbreviations used in this paper: AAF/PH, 2-acetylaminofluorene/partial hepatectomy; AH, adenomatous hyperplasia; CDAA, choline-deficient L-amino acid-defined; DAPI, 4′,6-diamidino-2-phenyliondole dihydrochloride; DIP1, D-type cyclin-interaction protein 1; GCIP, Grap2 cyclin-D interacting protein; GST-P, glutathione S-transferase placental form; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HES1, hairy and enhancer of split 1; HHM, human homologue of maid; HLH, helix-loop-helix; HNF4, hepatocyte nuclear factor 4; Jab1, Jun activation domain binding protein 1; Maid, murine maternal Id-like molecule; NHDF, normal human dermal fibroblast; RNAi, RNA interference.

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was found as a protein with increased expression during the 2-cell stage following fertilization and is believed to be closely involved in development.[7] The results of previous studies have also shown that expression of HHM in the fetal liver is higher than that in the adult liver, that HHM is expressed specifically in foci of the 2-acetylaminofluorene/partial hepatectomy (AAF/PH) model,[8] which is an animal model used to monitor the differentiation of rat hepatic stem cells, oval cells into hepatocytes, and that HHM regulates the activity of HNF4,[6] which is a hepatocyte differentiation factor, thus suggesting that HHM plays an important role in the development and differentiation of the liver.[6] HHM has been also cloned as D-type cyclin-interaction protein 1 (DIP1)[9] and Grap2 cyclin-D interacting protein (GCIP),[10] binding to cyclin D1 and Grap2, respectively. In this manner, HHM binds various proteins to express its biochemical functions.

As hepatitis B virus (HBV) and hepatitis C virus (HCV) infections become more common throughout the world, the number of patients with hepatocellular carcinoma (HCC) is increasing rapidly.[11–15] However, the gene that regulates hepatocarcinogenesis has not been identified. It has been clinically shown that chronic inflammation at the progression from chronic hepatitis to cirrhosis increases the risk for hepatocarcinogenesis.[16–18] In the present study, to assess the role of HHM in hepatocarcinogenesis, HHM expression in the choline deficient L-amino acid defined (CDAA) diet model was analyzed. Animals fed the CDAA diet develop glutathione S-transferase placental form (GST-P)-positive lesions, thought to be preneoplastic, after 12 weeks.[19] After 30 weeks, these lesions turn into HCCs, and, after 52 weeks, HCCs are seen in almost all rats.[20] Furthermore, HHM expression in human preneoplastic regions, adenomatous hyperplasia (AH), and HCC samples collected under the guidance of abdominal ultrasound were histologically assessed using HHM-specific antibodies. To analyze the function of HHM, a transcriptional regulatory protein, we tried to identify proteins that bind to HHM using a yeast 2-hybrid screen. We also analyzed HHM function by induction of HHM by adenovirus system or the knockdown of HHM by RNA interference (RNAi) targeting HHM.

Materials and Methods

Animal Model

Male Wistar rats (6 weeks old and weighing 140 to 150 g) were obtained from Nippon SLC Co., Ltd. (Shizuoka, Japan). CDAA diets were obtained in powdered form from Dyets, Inc. (Bethlehem, PA). Rats were housed under constant temperature (25°C), humidity, and lighting (12 hours light, 12 hours dark) conditions and were given the CDAA diets and water ad libitum for 48 weeks. This experiment was reviewed by the committee of the Ethics on Animal Experiment in Yamaguchi University School of Medicine and was performed under the Guidelines for Animal Experiment in Yamaguchi University School of Medicine.

In Situ Hybridization

The deletion mutant isoform of HHM cDNA probe was used for in situ hybridization (ISH). Plasmid pGEM-ΔHHM (1–184 aa) was made by inserting ΔHHM (1–184 aa) cDNA into the *Eco*RI/*Xba*I site of pGEM4 vector (Promega).[6] Sense and antisense digoxigenin-labeled probes were synthesized from pGEM-ΔHHM (1–184 aa). Antisense and sense (as a negative control) probes incorporating digoxigenin-UTP for ISH were made by in vitro transcription using SP6 or T7 RNA polymerase (Roche Molecular Biochemicals, Tokyo, Japan) after linearization of the plasmid. Briefly, after deparafinization and hydration, slides were treated with HCl, proteinase K (DakoCytomation, Kyoto, Japan), and Triton X-100 (Nacalai Tesque Inc., Kyoto, Japan). Hybridization was performed at 50°C. After a stringent wash and RNase A (Worthington Biochemical Corporation, Lakewood, NJ) treatment, the hybridized probe was revealed using a DAKO GenPoint System (DakoCytomation).

Production of Antibody Against HHM

The peptide ΔHHM (33–53 aa, accession number NP_036274 (HHM) is similar to rat homologue of Maid at 64–84 aa except for 75 aa in accession number XP_342503), which was chemically synthesized as an antigen by Medical & Biological Laboratories Co., Ltd. (MBL) (Nagoya, Japan). Antibodies against HHM were prepared by injecting 2 SPF Japanese white rabbits with ΔHHM (33–53 aa) 6 times. Because the immunoreactivity of antibodies against HHM was higher in 1 of the 2 rabbits, anti-HHM antibody (0.7779 mg/mL) (MBL) was obtained by subjecting the antibodies to affinity purification using ΔHHM (33–53 aa)-coupled Sepharose. This anti-HHM antibody was used for rat and human immunohistochemical analysis, immunoprecipitation assay, and fluorescent immunohistochemical examination.

Tissue Samples and Immunohistochemical Analysis

Seventy-eight liver tissue samples were obtained from 63 patients, who were admitted to Yamaguchi University hospital between 1999 and 2004, by ultrasonically guided needle biopsy for differential diagnosis (43 males, 20 females; mean age, 68.4 ± 8.4 years; range, 44–86 years). We used an ALOKA ProSound SSD-5500 ultrasound transducer and a 21-gauge needle (New Majima Needle) (Top. Co., Tokyo, Japan). The method of ultrasound-guided, fine-needle biopsy was previously described.[21,22] Histologic diagnoses were made according to the World Health Organization criteria by experienced liver specialists and pathologists. With approval from

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