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Sox9 expression in carcinogenesis and its clinical significance in intrahepatic cholangiocarcinoma

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ABSTRACT

Background: Intrahepatic cholangiocarcinomas develop through a multi-step carcinogenesis. Precancerous lesions are defined as biliary intraepithelial neoplasia. Sex determining region Y-box9 (Sox9) is required for the normal differentiation of the biliary tract.
Aims: To evaluate the Sox9 expression in carcinogenesis and its correlation with clinicopathological features in intrahepatic cholangiocarcinoma.
Methods: Sox9 expression in normal epithelium, biliary intraepithelial neoplasia, and intrahepatic cholangiocarcinoma were investigated immunohistochemically using 43 specimens of intrahepatic cholangiocarcinoma. Sox9 expression in intrahepatic cholangiocarcinoma was compared with the clinicopathological features. The molecular effects of Sox9 were investigated by gene transfection to intrahepatic cholangiocarcinoma cell lines.
Results: Sox9 expression was decreased from the normal epithelium to the biliary intraepithelial neoplasia in a stepwise fashion. In 51.2% (22/43) of the patients with intrahepatic cholangiocarcinoma, Sox9 expression was positive, and Sox9 expression was significantly associated with the biliary infiltration ($P=0.034$) and poor overall survival ($P=0.039$). Upregulation of Sox9 promoted the cell migration and invasion, and decreased the E-cadherin expression and increased the vimentin and α-SMA expression in cell lines.
Conclusions: Decreased Sox9 expression may be related to the early stage of the carcinogenesis of intrahepatic cholangiocarcinoma. Sox9 overexpression in intrahepatic cholangiocarcinoma is related to biliary infiltration and poorer prognosis, and it promotes cell migration and invasion, via the epithelial-to-mesenchymal transition.

1. Introduction

Intrahepatic cholangiocarcinoma (ICC) is an epithelial cancer of the intrahepatic biliary tract. The incidence of ICC is rare, but increasing worldwide [1]. The most effective treatment for an ICC is radical resection, but ICCs are often diagnosed at an advanced stage, at which surgery may not be possible [2–4]. The prognosis for ICCs is still poor despite the introduction of chemotherapy with gemcitabine or S-1 (tegafur, gimeracil, oteracil) and radiotherapy [3,5]. The elucidation of the mechanisms underlying the carcinogenesis of the biliary tract is a key to improving the prognosis of ICCs.

ICCs originate from epithelial cells of the intrahepatic biliary tract, typically in response to chronic inflammation, and they develop via a multi-step carcinogenesis [6]. Two types of premalignant lesions of cholangiocarcinoma have been identified. Flat or low papillary-type biliary intraepithelial neoplasia (BilINs) and papillary-type intraductal papillary mucinous tumours of the bile

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duct (IPN-Bs) are thought to progress to carcinoma via multistep processes [7,8]. BilINs are classified into three grades (BilIN-1, BilIN-2, and BilIN-3) according to the degree of atypia: BilIN-1 corresponds to low-grade dysplasia, BilIN-2, to intermediate-grade dysplasia, and BilIN-3, to high-grade dysplasia.

Sox9 (Sry [sex-determining region Y] – box9) is a member of the Sry-related high-mobility group (HMG) box family. Sox9 plays an important role not only in sex determination, but also in chondrogenesis and the embryonic formation of several tissues and organs, including the testis, heart, lung, pancreas, biliary tract and the central nervous system [9–15]. Abnormality in any of several genes that play a role in the normal differentiation of several organs can cause several neoplasms [16,17]. Several studies have found that digestive system cancers such as hepatocellular carcinoma (HCC), oesophageal cancer, gastric cancer (GC) and colorectal cancer are correlated with Sox9 expression in carcinogenesis [18–21]. Moreover, Sox9 has attracted attention as a progressive or prognostic biomarker in patients diagnosed with HCC or GC [18,22].

In recent years, we have reported that Sox9 expression gradually decreased in the carcinogenesis of intraductal papillary mucinous neoplasm (IPMN) in the pancreas, and that Sox9 expression in IPN-B, which is recognised as the counterpart of IPMN, was significantly lower than the expression in the normal epithelium of the bile duct [23,24]. In addition, a stepwise decrease of Sox9 expression in pancreatic intraepithelial neoplasia, which is recognised as the counterpart of BilIN, was reported by Shroff et al. [25]. In the present study, we analysed Sox9 expression in ICCs including the precancerous lesions, and we determined this expression's correlation with clinicopathological features. We also investigated the molecular effects of Sox9 expression on ICC cell lines, especially in cell migration and invasion.

2. Materials and methods

2.1. Tissue samples and immunohistochemistry

A total of 43 patients [male/female: 26/17, median age (range): 71 (23–88) years] diagnosed with ICC underwent hepatic resection of varying extents in our department between April 2000 and October 2014. The patients' characteristics are shown in Table 1. Tumour staging was classified according to the TNM Classification of Malignant Tumours defined by the International Union Against Cancer (seventh edition) [26]. Biliary infiltration was defined as positive in cases in which the cancer cells had invaded the intrahepatic bile duct. None of the patients received any chemotherapy or radiation therapy before their operation. Surgical specimens were investigated immunohistochemically for Sox9 expression. This study was approved by the Ethics Committee of Nagasaki University, and conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from each patients included in this study.

A total of 43 cancerous lesions and 43 normal biliary epithelium samples, one of each from each patient, were investigated. In addition, a total of 47 BilIN-1 lesions, 27 BilIN-2 lesions, and 16 BilIN-3 lesions were obtained from 23 ICC surgical specimens under a microscope. The investigated normal epithelium and BilIN samples were selected from tissue outside of the main cancerous area and obtained from the periphery of the liver.

Immunohistochemistry was performed using mouse monoclonal anti-Sox9 antibody (Abcam, Cambridge, UK) following the protocol described by Tanaka et al. [23]. The quantification of Sox9 expression was performed using an immunoreactive score (IRS) for each lesion, taking into account the percentage (0, 0%; 1, 1–10%; 2, 11–50%; 3, 51–100%) and the intensity (negative: 0, weak: 1, moderate: 2, strong: 3) of stained cells. Strong and moderate staining could be detected at ×40 and ×100 magnification, respectively, whereas weak staining could be detected at ×200 magnification. The IRS (0–9) was obtained by multiplying the percentage score and the intensity score. An IRS ≥ 1 was defined as Sox9-positive, and the IRS = 0 was defined as Sox9-negative. The correlations between Sox9 expression and the clinicopathological features were investigated. Overall survival was calculated from the day of operation. Two patients who did not undergo curative resection were excluded from the overall survival analysis.

2.2. Cell line culture, transfection and RNA interference

Human ICC cell lines HuCCT1 and HuH28 were purchased from JCRB Cell Bank, Japan. HuCCT1 and HuH28 cells were grown at 37 °C under 5%CO_2 in RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin (Gibco®; Invitrogen, Carlsbad, CA). The Sox9 cDNA ligated into the pCMV6 vector was purchased from OriGene (Rockville, MD). As a negative control (Mock), a pCMV6 vector with no insertion was used.

HuCCT1 and HuH28 cells were transfected with Sox9 cDNA or pCMV6 vector using TransIT®-2020 Transfection Reagent (Mirus, Madison, WI) according to the manufacturer's instructions. HuCCT1 cells were transfected with Sox9 short interference RNA (siRNA) (Silencer® Select siRNA, Ambion, Carlsbad, CA) using Lipofectamine® RNAiMAX Reagent (Invitrogen) according to the manufacturer's instructions. As a negative control (scramble), Silencer® Select Negative Control siRNA (Ambion) was used. Successful regulation of Sox9 expression in the HuCCT1 and HuH28 cells was verified by Western blotting.

2.3. Western blotting

The samples were lysed with Triton-deoxycholate (DOC) lysis buffer. After centrifugation, the supernatant was stored at −80 °C until analysis. After the amounts of protein were measured using a BCA protein assay kit (Pierce 23227, Life Technologies, Carlsbad, CA), loading buffer [2% SDS, 5% β-mercaptoethanol, 5% sucrose, 0.005% bromophenol blue and 62.5 mM Tris–HCl (pH6.8)] was added to the proteins, and the mixtures were incubated at 95 °C for 5 min. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 12% acrylamide gels. The proteins were transferred onto Immobilon-P membranes (IPVH10100, Millipore, Bedford, MA) in a transfer buffer containing 20% methanol.

After blocking in 5% skim milk for 60 min at room temperature, the membranes were reacted with primary antibody overnight at 4 °C, and followed by horseradish peroxidase (HRP)-conjugated secondary antibodies. The following primary antibodies were used: Sox9 (ab3697, Abcam), E-cadherin (ab15148, Abcam), vimentin and α-SMA: (ab157392, Abcam). The proteins were visualised using a chemiluminescence system (RPN2132, GE Healthcare, Buckinghamshire, UK). The quantification of the Western blotting data was performed with GAPDH as a reference, using Image J software (NIH, Bethesda, Maryland).

2.4. Cell proliferation assay

Twenty-four, 48, and 72 hours after the transfection of HuCCT1 and HuH28 cells, Cell Proliferation Reagent WST-1 (Roche Applied Science, Indianapolis, IN) was used according to the manufacturer's instructions. A microplate reader (Multiskan® FC, Thermo Fisher Scientific, Vantaa, Finland) was used to calculate the cleavage of WST-1 to formazan by metabolically active cells.