Human Pathology (2013) 44, 1031–1037

ELSEVIER

Human PATHOLOGY

www.elsevier.com/locate/humpath

Original contribution

Different roles of inducible nitric oxide synthase and cyclooxygenase-2 in carcinogenesis and metastasis of intrahepatic cholangiocarcinoma

Shinichi Aishima MD, PhD[a,*], Yohei Mano MD[b], Yuki Tanaka MD[a], Yuichiro Kubo MD[a], Ken Shirabe MD, PhD[b], Yoshihiko Maehara MD, PhD[b], Yoshinao Oda MD, PhD[a]

[a]*Department of Anatomic Pathology, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan*
[b]*Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan*

Received 2 August 2012; revised 6 September 2012; accepted 7 September 2012

Keywords:
iNOS;
COX;
VEGF-C;
Lymph node metastasis

Summary Inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) have been implicated in chronic inflammatory conditions and carcinogenesis. However, little is known about the biological significance of iNOS and COX-2 in cholangiocarcinoma or its precursors or metastatic lesions. We examined iNOS and COX-2 immunohisotochemical expression in 40 biliary intraepithelial neoplasias, 134 primary intrahepatic cholangiocarcinoma cases, and 27 metastatic lymph nodes and analyzed the correlations with grade of atypia of biliary intraepithelial neoplasia, clinicopathological factors and outcomes of intrahepatic cholangiocarcinoma. iNOS and COX-2 expression was highly expressed in reactive epithelium and biliary intraepithelial neoplasia. In intrahepatic cholangiocarcinoma, lymphatic invasion and lymph node metastasis were significantly correlated with negative iNOS expression (P = .0002, P = .0324, respectively) and positive COX-2 expression (P = .0012, P = .0063, respectively). Vascular endothelial growth factor–C expression was associated with COX-2 expression (P = .0053), but not with iNOS expression. COX-2 expression in primary intrahepatic cholangiocarcinoma was higher than that in metastatic lymph nodes (P < .0001). COX-2-positive expression indicated a poor intrahepatic cholangiocarcinoma outcome (P = .0273). This study indicates that iNOS and COX-2 may play roles in carcinogenesis via biliary intraepithelial neoplasia, but play different roles in metastasis of intrahepatic cholangiocarcinoma. COX-2 may participate in a higher lymphatic invasion and metastasis via the vascular endothelial growth factor–C pathway.

1. Introduction

Intrahepatic cholangiocarcinoma originates from the intrahepatic bile duct and is associated with an extremely poor prognosis. Conditions such as hepatolithiasis, primary sclerosing cholangitis, and liver fluke infection involving chronic inflammation of the biliary trees may be major risk factors for cholangiocarcinoma arising from large bile duct [1]. The development of intrahepatic cholangiocarcinoma is also known to be related to chronic hepatitis, alcoholic liver disease, and the cirrhosis associated with these diseases [2,3]. These chronic inflammatory conditions, as well as viral or parasitic infections, induce the production of reactive oxygen species and reactive nitrogen species, which may play a key role in carcinogenesis [4,5].

* Corresponding author.
E-mail address: saish@surgpath.med.kyushu-u.ac.jp (S. Aishima).

0046-8177/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.humpath.2012.09.004

Nitric oxide (NO) is generated by inducible nitric oxide synthase (iNOS) of the epithelial cells and inflammatory cells in inflamed tissue [4–6]. Inflammatory cytokines induce iNOS, and the production of NO causes DNA damage in cholangiocarcinoma cells [7] and the biliary epithelium of primary sclerosing cholangitis [8]. iNOS binds to cyclooxygenase-2 (COX-2) and enhances COX-2 activity [9]. COX-2 catalyzes the rate-limiting step of prostaglandin synthesis and is an early-response gene that is thought to be induced by either growth factor or oncogenes. The COX-2-derived prostaglandin pathway regulates cholangiocarcinoma growth, and this signaling pathway is considered as a potential therapeutic target [10]. Bile acids can induce COX-2 in cholangiocarcinoma cells [11], and COX-2 has been reported to be overexpressed in chronic cholangitis, primary sclerosing cholangitis, and cholangiocarcinoma [12–15]. These results suggest that both iNOS and COX-2 play important roles in cholangiocarcinogenesis.

Invasive cholangiocarcinomas arising from the large bile duct are often preceded by distinct precursor or non-invasive biliary lesions, including biliary intraepithelial neoplasia (BilIN), that is, dysplastic epithelium with flat or micropapillary growths [16,17]. Intrahepatic cholangiocarcinoma is divided into perihilar and peripheral forms based on its location [18], and multistep carcinogenesis from dysplasia to adenocarcinoma has been proposed for perihilar intrahepatic cholangiocarcinoma [18,19].

We have previously investigated vascular endothelial growth factor-C (VEGF-C) expression in intrahepatic cholangiocarcinoma and indicated that lymphatic invasion of intrahepatic cholangiocarcinoma was related to VEGF-C [20]. COX-2 can induce lymphangiogenesis in cancer by the induction of VEGF-C [21].

Protein expression of iNOS and COX-2 has been detected in cholangitis and cholangiocarcinoma, but expression levels of these proteins in the context of neoplasms such as BilIN, that is, in precancerous lesions of the biliary tree, have not yet been fully investigated. In addition, the status of iNOS and COX-2 expression in metastatic lesions from cholangiocarcinoma remains unclear. Therefore, we examined the roles played by iNOS and COX-2 in cholangiocarcinogenesis, tumor progression, an association of VEGF-C expression, and metastasis of intrahepatic cholangiocarcinoma.

2. Materials and methods

2.1. Tissue samples

Intrahepatic cholangiocarcinomas and tissues exhibiting BilIN were surgically resected and diagnosed at the Department of Anatomic Pathology of Kyushu University from 1985 to 2010. Our study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. For strict privacy protection, identifying information for all samples was removed before analysis. One hundred thirty-four cases of intrahepatic cholangiocarcinoma showed invasive carcinoma without special histologic features such as sarcomatoid, adenosquamous, or signet-ring cells. We selected biliary reactive epithelia (n = 16, 11 hepatolithiasis and 5 primary sclerosing cholangitis) and BilINs (n = 40, 32 hepatolithiasis and 8 primary sclerosing cholangitis). Reactive epithelium was defined as mildly hyperplastic epithelium with inflammatory cell infiltration, but lacking a loss of cell polarity. BilIN-1 showed mild cellular atypia with enlarged nuclei and only a minimal disturbance of cell polarity (n = 20). BilIN-2 showed evident cellular atypia with a focal disturbance of cell polarity (n = 12). BilIN-3 showed definite cellular atypia, or nuclear pleomorphism, or complete loss of cell polarity (n = 8). BilIN lesions were graded based on the histopathological definitions of BilIN [22]. The median age of the patients with intrahepatic cholangiocarcinoma was 64 years (range, 33-90 years); the intrahepatic cholangiocarcinoma group included 82 men and 52 women. Hepatitis B surface antigen (HBsAg) or hepatitis C virus antibody (HCVAb) was positive in 40 cases. Vascular invasion, lymphatic invasion, and lymph node metastasis were observed in 86 cases (64%), 64 cases (48%), and 34 cases (25%) of intrahepatic cholangiocarcinoma, respectively. Metastatic lymph nodes were examined in 27 cases.

2.2. Immunohistochemistry and evaluation

Tissue samples were fixed with 10% formaldehyde, embedded in paraffin, and sectioned into 4-μm-thick serial slices. Immunohistochemical staining was performed by the streptavidin-biotin-peroxidase method (Histofine; Nichirei, Tokyo, Japan). The primary antibodies used in this study were mouse monoclonal anti-iNOS (1:200 dilution; BD Biosciences), goat polyclonal anti-COX2 (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), goat polyclonal anti-VEGF-C (1:200 dilution; Santa Cruz Biotechnology), and mouse monoclonal anti-ki-67 (1:100; DAKO, Carpinteria, CA). After the inhibition of endogenous peroxidase in a 3% H_2O_2-methanol solution for 20 min and antigen retrieval (microwave irradiation in citrate buffer [pH 6.0] for iNOS, COX2, and ki-67 antibodies), sections were exposed to each primary antibody at 4°C overnight. The sections for VEGF-C were digested with 0.1% trypsin solution at 37°C for 30 min. The sections were then reacted in 3,3′-diaminobenzidine, counterstained with hematoxylin, and mounted. Evaluation of the immunohistochemical results was scored by 2 pathologists (Y.T. and S.A.) without knowledge of the clinical data. Immunohistochemical staining for iNOS and COX-2 was evaluated in the area showing the highest degree of dysplasia in each BilIN and intraductal papillary neoplasm of the bile duct. To evaluate the expression of intrahepatic cholangiocarcinoma (ICC), representative fields including both cancerous and non-cancerous areas were selected. The proportion of positive cells was semiquantitatively measured using the following