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Original article

KRAS mutation and immunohistochemical profile in intraductal papillary neoplasm of the intrahepatic bile ducts

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ABSTRACT

Intraductal papillary neoplasm of bile duct (IPNB) is characterized by a spectrum of diseases ranging from low-grade intraepithelial neoplasia to invasive carcinoma. In the present study, we aimed to investigate immunophenotypic features and *KRAS* mutations in relation to pathological subtypes and grades in Chinese patients with IPNBs. A total of 46 patients with IPNBs and 11 invasive adenocarcinomas arising in IPNBs (invasive IPNBs) were enrolled and clinicopathological data were analyzed. It was found that CK7 was expressed in 42 of the 46 neoplastic lesions. HepPar1 was expressed in 11 of the 46 noninvasive IPNBs, but not in invasive IPNBs. Additionally, CK19 was frequently expressed in both noninvasive IPNBs and invasive IPNBs. The intestinal-type IPNBs had a significantly higher percentage of MUC2 expression relative to the pancreaticobiliary ($P = 0.015$) and gastric-type IPNBs ($P < 0.001$). High-grade IPNBs and invasive IPNBs showed increased expression of cyclin D1, Ki-67, p53, mCEA, and CA19-9. The rate of *KRAS* mutation was significantly higher in high-grade IPNBs ($P = 0.001$) and invasive IPNBs ($P = 0.006$) than that in low- to intermediate-grade IPNBs. Additionally, *KRAS* mutation was significantly associated with tumor size, and Ki-67 expression. In conclusion, the expression of cyclin D, Ki-67, p53, mCEA and CA19-9 and *KRAS* mutation status are significantly correlated with histological grades of IPNBs.

1. Introduction

Intrahepatic cholangiocarcinoma (ICC) is the second most common cancer occurring in the liver and accounts for 10–20% of all deaths from hepatobiliary malignancies worldwide [1]. According to the WHO classification (2010), two entities are considered as precursor lesions of ICC: biliary intraepithelial neoplasia and intraductal papillary neoplasms [2]. Intraductal papillary neoplasm of bile duct (IPNB) is characterized by dilated intrahepatic bile ducts filled with papillary or villous tumor covered by well-differentiated neoplastic epithelium [3–5]. Papillary neoplasms including papillary carcinoma and papillomatosis represent 4–10% of all biliary epithelial neoplasms [2,6]. IPNB is a spectrum of diseases ranging from low-grade intraepithelial neoplasia to invasive carcinoma [2,6]. It is challenging to make a definite diagnosis of non-invasive IPNBs with high-grade dysplasia because they often show atypical pathological features.

Several clinical and animal studies have described a number of genetic alterations during the progression of intraductal papillary neoplasms. *KRAS* mutations have been found in biliary intraepithelial neoplasia, IPNB, and cholangiocarcinoma [7–11]. It is estimated that IPNB has a *KRAS* mutation rate of 18–46% [3]. In the present study, we enrolled a large series of Chinese patients with IPNB and investigated immunophenotypic features and *KRAS* mutations in relation to pathological subtypes and grades.

2. Materials and methods

2.1. Case selection and histological classification

The study protocol was approved by the Ethics Committee of Second Military Medical University (Shanghai, China), and written informed consent was obtained from each patient. Surgically resected specimens were obtained from the Eastern Hepatobiliary Surgery Hospital of Second Military Medical University (Naval Medical University) between January 2013 and December 2016. Descriptions like “papillomatosis” or “polypoid” were included during the search. All cases were confirmed by pathological examination. Based on the WHO classification of tumors of the digestive system [2], IPNB was defined as a biliary tumor consisting predominantly of non-invasive neoplastic cells, with an intraductal papillary architecture. IPNB cases were further classified into the following subtypes: pancreatobiliary, intestinal, gastric, and oncocytic [12,13]. The degree of epithelial dysplasia was classified into

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low, intermediate and high grades according to WHO criteria [2], Invasive IPNB was defined as a biliary tumor consisting of non-invasive intraductal papillary neoplastic cells and invasive components [2].

2.2. Immunohistochemistry

All samples were fixed in 10% buffered formalin and embedded in paraffin. Tissue sections were deparaffinized, rehydrated, and subjected to elimination of endogenous peroxidase activity with 3% H_2O_2. After blocking with 5% normal mouse serum for 15 min, tissue sections were incubated with the following primary antibodies against mucin core protein (MUC) 1, MUC2, MUC5AC, MUC6, cytokeratin (CK) 7, CK20, CK19, Hep Par 1, β-catein, Cyclin D, ki67, p53, CEA, and CA19-9 (Supplementary Table S1). After washing, sections were incubated with biotinylated goat anti-mouse IgG for 30 min at room temperature. 3,3′-diaminobenzidine was used as a chromogen. The sections were counterstained with hematoxylin before microscopic examination. Substitution of primary antibodies by nonimmune serum was used as negative controls. The immunostaining results were evaluated semi-quantitatively by two independent pathologists in a blind fashion. The cases with 25% or more of cells stained were defined as positive and those with less than 25% negative [14].

2.3. DNA extraction

Tissues corresponding to the noninvasive IPNB was microdissected from all 46 cases, noninvasive and invasive component of invasive IPNB specimens from 11 cases. In brief, paraffin-embedded tissue sections (10 μm) were deparaffinized, dehydrated, pre-stained with hematoxylin, and then dehydrated in graded alcohol. Microdissection was performed under the dissection microscope with a laser capture microdissector (Leica AS LMD system, Leica, Wetzlar, Germany). DNA was extracted using QIAamp® DNA formalin-fixed paraffin-embedded (FFPE) Tissue Kit (Qiagen, Hiden, Germany) following the manufacturer's instructions. DNA quality and quantity was measured by ultraviolet spectrophotometry. DNA samples were stored at −20 °C until use.

2.4. KRAS mutation analysis

The AmoyDx® *KRAS* mutation detection kit (Amoy Diagnostics Ltd., Fujian, China) was employed to measure *KRAS* mutations, according to the manufacturer's protocol. The genomic sites examined by PCR are listed in Supplementary Table S2. A negative control without the DNA template was included. If the amplification curve was not the classic S-curve or the cycle threshold (Ct) value was > 30, the result was considered as wild type.

2.5. Statistical analysis

Statistical differences were determined using the SPSS 13.0 software (SPSS, Chicago, IL, USA). Categorized data were analyzed by paired chi-square (χ^2) test, Spearman correlation test, and Fisher's exact test (2-sided). Continuous data are expressed as mean ± standard deviation and were analyzed using the Student's *t*-test. All P values < 0.05 were considered statistically significant.

3. Results

3.1. Clinicopathologic characteristics

Clinical and pathologic characteristics of 46 noninvasive IPNBs and 11 invasive IPNBs are showed in Supplementary Table S3. The median age of the 57 patients enrolled was 60 years (range, 35–81 years). Twelve (21.1%) of the 57 patients were asymptomatic and detected incidentally. Thirteen patients (22.8%) had an intrahepatic duct stone on radiological imaging. In all the noninvasive IPNB cases, the neoplasms were in direct connection with the intrahepatic biliary tree, including 30 cases with the predominant intrahepatic bile duct involvement, and 16 cases with involvement of both intrahepatic and extrahepatic ducts. Noninvasive IPNBs had a significantly shorter median diameter than invasive IPNBs (2.9 vs. 4.5 cm; $P = 0.008$). Six of the 46 patients had low-grade, 20 moderate-grade, and 20 high-grade dysplasia. Six of 11 invasive IPNBs exhibited tubular carcinomas. Of the remaining 5 cases, 3 cases showed invasive mucinous carcinoma, and 2 showed papillary carcinomas. All invasive cases had high-grade dysplasia or so-called *in situ* cancer in the non-invasive components.

Table 1
Immunohistochemical profiles in noninvasive and invasive IPNBs.

		Noninvasive IPNB (n = 46)	Noninvasive component of invasive IPNB (n = 11)	Invasive component of invasive IPNB (n = 11)
Hep Par 1	+	11	0	0
	–	35	11	11
CK7	+	42	10	10
	–	4	1	1
CK20	+	9	1	1
	–	37	10	10
CK19	+	40	10	10
	–	6	1	1
MUC1	+	22	9*	**9***
	–	24	2	2
MUC2	+	17	3	3
	–	29	8	8
MUC5AC	+	43	11	11
	–	3	0	0
MUC6	+	30	9	9
	–	16	2	2
Membranous β-catenin	+	13	5	5
	–	33	6	6
Cyclin D	+	23	8	8
	–	23	3	3
ki67	+	22	8	**9***
	–	24	3	2
p53	+	8	4	**6***
	–	38	7	5
mCEA	+	6	**7***†	**10***†
	–	40	4	1
CA19-9	+	13	7*	**10***†
	–	33	4	1

*P < 0.05 *vs.* Noninvasive IPNB. †P < 0.05 *vs.* Noninvasive component of invasive IPNB. Bold values indicate significance after Bonferroni's correction.

3.2. Expression of HepPar1, CK7, CK19 and CK20

The results of HepPar1, CK7, CK19 and CK20 expression are shown in Tables 1 and 2 and Fig. 1. CK7 was expressed in 42 of the 46 neoplastic lesions and the surrounding non-neoplastic biliary ductal epithelium. In contrast, CK20 was more frequently detected in intraductal components (9/46) than in invasive components of IPNBs (1/11; $P = 0.668$). However, CK20 was not expressed in non-neoplastic biliary duct epithelium. The intestinal type tumors commonly expressed CK20 (8/14), whereas only one gastric type, but not the pancreatobiliary type or oncocytic type had CK20 expression. HepPar1 was expressed in 11 of the 46 noninvasive IPNBs, whereas no HepPar1 expression was found in invasive cancer cases. Additionally, CK19 was frequently expressed in both intraductal components (41/46) and invasive components of IPNBs (9/11).

3.3. Expression of MUC1, MUC2, MUC5AC and MUC6

As shown in Fig. 2, MUC1 was expressed mainly in the apical membrane in IPNBs and in the cytoplasm in invasive tumors. In