Evaluation of Intraductal Ultrasonography, Endoscopic Brush Cytology and K-ras, P53 Gene Mutation in the Early Diagnosis of Malignant Bile Duct Stricture

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Abstract

Background: In qualitative diagnosis of bile duct stenosis, single diagnostic measure is difficult to make a correct diagnosis, to combine several diagnostic techniques may be helpful to make an accurate diagnosis. The aim of this study was to evaluate the value of intraductal ultrasonography (IDUS), endoscopic brush cytology and K-ras, P53 gene mutation in the early diagnosis of malignant biliary stricture.
Methods: From February 2012 to February 2013, 84 patients with suspected malignant biliary stricture were performed IDUS firstly, then endoscopic brush cytology and finally K-ras, P53 gene mutation detection, the sensitivity, specificity, positive predictive value, negative predictive value and accuracy of all above ways were evaluated and compared.
Results: Of 84 patients, 52 cases were ultimately diagnosed malignant biliary stenosis; of which, 9 cases had no recurrence or metastasis to other organs after radical operation during the follow-up period. IDUS combined with brush cytology and K-ras + P53 gene mutation detection had obvious advantage in the sensitivity, accuracy and negative predictive value than any other joint detection and single detection (the advantage was more significant compared with IDUS + brush cytology or any single detection $P < 0.01$). There were obvious statistical significance in the sensitivity and accuracy between IDUS + brush cytology + P53 or IDUS + brush cytology + K-ras and IDUS + brush cytology or IDUS ($P < 0.05$). There was no statistical significance in the sensitivity, specificity, positive predictive value, negative predictive value and accuracy between IDUS + brush cytology + P53 and IDUS + brush cytology + K-ras ($P > 0.05$).
Conclusions: IDUS combined with brush cytology and K-ras, P53 gene mutation detection is better than the separate detection and contribute to the early diagnosis of malignant biliary stricture. Its more widespread use is recommended.

Key words: Brush Cytology; Intraductal Ultrasonography; K-ras; P53 Gene Mutations; Malignant Biliary Stricture

Introduction

Malignant biliary stenosis is a common clinical disease. Because of the atypical early symptoms, low radical resection rate, and poor prognosis, the early diagnosis of malignant biliary stenosis is crucial.[1] But the sensitivity and specificity of bile, blood tumor markers[2] in diagnosis of malignant biliary strictures were lower, while sometimes it is very difficult for us to determine early the nature of benign and malignant biliary stenosis according to tumor markers, ultrasound, computed tomography (CT), magnetic resonance cholangiopancreatography (MRCP), percutaneous transhepatic cholangio drainage and other imaging examination. And the implantation of biliary metal stent or operation for those patients with a vague diagnosis could often lead to medical disputes. Therefore, the clinical research for qualitative diagnosis of bile duct stricture has very high value. During the period from February 2012 to February 2013, 84 cases with suspected malignant stricture of bile duct were diagnosed satisfactorily through intraductal ultrasonography (IDUS), endoscopic brush cytology and K-ras, P53 gene mutation detection, and results are reported as follows now.

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Methods

Patients

From February 2012 to February 2013, 84 patients with suspicious malignant biliary stricture (cholangiocarcinoma) were diagnosed in our Endoscopy Unit at the First People's Hospital of Hangzhou, China. The blood tumor markers, MRCP or CT and other clinical data of all patients supported the diagnosis of malignant biliary stenosis. Inclusion

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criteria: (1) Symptoms or signs in accordance with biliary tract disease; (2) Suspected malignant biliary stricture according to imaging clinical data; (3) Unrestricted age and gender; (4) Having indications for endoscopic retrograde cholangiopancreatography (ERCP). Exclusion criteria: (1) Severe varicose gastroesophageal varices; (2) Severe outlet stenosis of stomach or duodenum; (3) Due to cardiac, pulmonary or renal insufficiency, patients cannot tolerate endoscopic operators; (4) The malignant tumor found in other organ besides bile duct; (5) Refused to participate in the study.

All patients or their spouses gave written informed consent for the procedures after receiving an explanation of the risks and benefits of IDUS and endoscopic brush cytology, the alternatives, and associated therapeutic procedures.

Materials

These included a TJF240/260 duodenoscope (Olympus, Japan), an APC300/ICC200EA high-frequency generator (Erbe, Tübingen, Germany), needle knife, papillotome, guide wire, catheter, basket, balloon, nasobiliary drainage tube, plastic stent, UMG20-29R ultrasound probe(Olympus, Japan), cell brush, K-ras and P53 gene mutation detection kit and so on.

Procedures

All procedures were performed by experienced operators. Preoperative pethidine and midazolam were used for analgesia and sedation and intravenous hyoscine hydrobromide for duodenal relaxation in every patient, with monitoring of arterial oxygen saturation and pulse rate. All patients were positioned in either the left lateral or semi-prone position to avoid any respiratory compromise.

All patients were performed IDUS, then brush cytology during ERCP, and specimens were sent to genetics laboratory for K-ras, P53 gene mutation detection. The main steps for K-ras, P53 gene mutation detection were as follows: (1) Extraction, primer synthesis of DNA in specimen; (2) Polymerase chain reaction (PCR) amplification; (3) Electrophoresis of PCR products; (4) The sequence of PCR products and analysis of the results.

Evaluation standard for benign or malignant bile duct stenosis

Bile duct benign, malignant stenosis judgment standard.[3] (1) Malignant stenosis: Confirmed by pathology (brush cytology or operation specimens) or malignancy supported by imaging data or the clinical progression during the follow-up; (2) Benign stricture: Confirmed by surgical pathology or brush cytology and followed-up at least for 12 months or more, no malignant syndrome appeared.

Intraductal ultrasonography image interpretation

All IDUS images were interpreted by two experienced endoscopic ultrasound experts. Criteria for malignant biliary stenosis:[3] Destruction of normal bile duct wall structure, visible irregular edge of hypoechoic mass on bile duct wall, uneven internal echo, the infiltrated surrounding tissue. Criteria for benign bile duct stenosis: Basic integrity three layer structure of bile duct wall, uniform echo, smooth edge; or hyperechoic full-thickness uniform thickening bile duct wall, no obvious hypoechoic mass and vascular invasion signs.

Clinical variables assessed

The following variables were recorded and compared: The sensitivity, specificity, positive predictive value, negative predictive value and accuracy of IDUS, brush cytology and K-ras, P53 gene mutation detection in early diagnosis of malignant bile duct stricture. The above indicators calculated by the following formula: Sensitivity = true positive/(true positive + false negative), specificity = true negative/(true negative + false positive), positive predictive value = true positive/total positive, negative predictive value = true negative/total negative, accuracy = (true positive + true negative)/total cases.

Statistical analysis

Categorical variables were summarized by frequencies and percentages and compared using Fisher's exact tests or Pearson's χ^2 test. A Statistical Software Package (SPSS, version 13.0; Chicago, IL, USA) was used for data management and analysis. A two-tailed *P* value below 0.05 was considered as statistically significant.

Results

Patient characteristics

Eighty-four patients were included in the present study, and of which, 52 cases were ultimately diagnosed malignant biliary stenosis, 32 cases benign biliary stricture by endoscopy or operation and the follow-up for more than 12 months. Their median age was 67 years (range, 46–98 years). The characteristics of all patients are shown in Table 1.

Intraductal ultrasonography

Of 52 cases with malignant biliary stricture, 33 cases were characterized as malignant biliary stricture by IDUS: Twenty-seven cases showed hypoechoic prominent lesions of the bile duct lumen and bile duct hierarchy disappeared, 6 cases showed the bile duct wall full-thickness, unclear hierarchy or interruption, of which 5 cases accompanied with

Table 1: The characteristics of all patients

Items	Malignant	Benign
Patients (*n*)	52	32
Age (years)	52 ± 4.58	45 ± 6.72
Male/female	24/28	17/15
Hilar bile duct (*n*)	20	14
Middle and distal bile duct (*n*)	32	18
Diagnostic routine (*n*)		
IDUS	33	25
Brush cytology	28	32
K-ras gene mutation	20	32
P53 gene mutation	22	32

IDUS: Intraductal ultrasonography.

peripheral enlarged lymph nodes. Of 32 cases with benign biliary stricture, seven were misdiagnosed as malignance due to fuzzy bile duct hierarchy, the other showed uniformly thickened bile duct wall and clear hierarchy, without peripheral enlarged lymph nodes, and 4 cases with visible stones. The sensitivity, specificity, positive predictive value, negative predictive value and accuracy of IDUS in diagnosing malignant bile duct stricture were 63.46% (33/52), 78.13% (25/32), 82.50% (33/40), 56.82% (25/44) and 69.05% (58/84).

Brush cytology

Of 52 cases with malignant biliary stricture, 28 cases were found visible cancer or highly atypical cells on brush cytology smear or liquid-based cytology, 16 cases found atypical cells, 8 cases negative, and the 24 cases were eventually diagnosed as malignant tumor. The sensitivity, specificity, positive predictive value, negative predictive value and accuracy of brush cytology in diagnosing malignant bile duct stricture were 53.85% (28/52), 100% (32/32), 100% (28/32), 57.14% (32/56) and 71.43% (60/84), while corresponding 65.38% (34/52), 84.38% (27/32), 87.18% (34/39), 60.00% (27/45) and 72.62% (61/84) in brush cytology + IDUS.

K-ras, P53 gene mutation detection

In the detection of brush cytology specimens, gene mutation exists only if one gene mutate among K-ras gene exon 1 and P53 exon 5, 6, 7, 8. The amplification success rate of K-ras gene exon 1 and P53 exon 5, 6, 7, 8, was 100%. The abnormal electrophoresis showed abnormal surge strips, increased and missing strips when K-ras gene exon 1 or P53 gene exon 5, 6, 7, 8 mutated.

In 52 cases with malignant biliary stricture, K-ras or P53 gene mutation was found in 20 cases or 22 cases, accounting for 38.46% and 42.31%. By DNA sequence, K-ras gene mutation was found mostly at codon 12, 6 cases at 6 and 36 codons. Its most mutation way was according to GGT-GAT, sometimes GGT-GTT, AGT-TGT. Point mutation was found in the vast majority of all patients, insertional mutagenesis in 4 cases. To P53 gene mutation, 3 cases in exon 5, 4 cases in exon 6, 4 cases in exon 7, 11 cases in exon 8, the mutation rate in exon 8 was higher ($P < 0.05$). P53 gene mutation had many forms and multiple mutation hot spots, but most of the mutation types were G-A and T-C.

K-ras, P53 gene mutation rate were 30% (6/20), 40% (8/20) respectively in hilar bile duct malignant stricture brush specimens, while corresponding 43.75% (14/32), 43.75% (14/32) in the lower bile duct. K-ras or P53 gene mutation rate was not related to lesion location in malignant biliary stricture brush specimens. These results suggest the high diagnostic specificity of K-ras, P53 gene mutations in malignant bile duct stenosis. K-ras gene mutation occurred in those patients with malignant biliary stricture comprising three papillary carcinoma, four high differentiated adenocarcinoma, five differentiated adenocarcinoma, five low differentiated adenocarcinoma, three undifferentiated adenocarcinoma, while corresponding, 4, 4, 5, 6 and 3 cases in patients with P53 gene mutation. Those suggest that K-ras, P53 gene mutation have nothing to do with histologic type of cholangiocarcinoma. K-ras, P53 gene mutation rate were, respectively, 38.89% (7/18) and 44.44% (8/18), in phase I–II cholangiocarcinoma, while corresponding 38.24% (13/34) and 41.18% (14/34) in phase III–IV cholangiocarcinoma. There was no significant difference between the two groups. It suggests that K-ras, P53 gene mutation have nothing to do with cholangiocarcinoma stages, which will help us to diagnose the cholangiocarcinoma earlier.

Comparison of the diagnosis value of single detection

Intraductal ultrasonography was superior to K-ras and P53 gene mutation detection on the sensitivity ($P < 0.05$) while IDUS had the more obvious advantage than K-ras mutation detection. There was no significant difference in the sensitivity between IDUS and brush cytology, or between K-ras and P53 gene mutation detection or between brush cytology and K-ras, P53 gene mutation detection, and there were significant difference in the specificity and positive predictive value of brush cytology or K-ras or P53 gene mutation detection and IDUS ($P < 0.05$); the negative predictive value was not significantly different; the accuracy of brush cytology had obvious advantages than K-ras gene mutation detection($P < 0.05$), no significant difference than others [Table 2].

Comparison of the diagnosis value of combined detection

Intraductal ultrasonography combined with brush cytology + K-ras + P53 had obvious advantage in the sensitivity than the other combined groups and any single detection ($P < 0.05$) (the advantage was more significant compared with IDUS + brush cytology and single detection, $P < 0.01$), and in the negative predictive value and accuracy than IDUS + brush cytology + K-ras ($P < 0.05$) (than IDUS + brush cytology, $P < 0.01$), and the advantages were obvious compared with IDUS + brush cytology + P53, but not statistically significant; There was not significantly different in the specificity and positive predictive value between IDUS + brush cytology + K-ras + P53 and the other any groups. There were obvious statistical significance in the sensitivity and accuracy between IDUS + brush cytology + P53 or IDUS + brush cytology + K-ras and IDUS + brush cytology or IDUS ($P < 0.05$), but not in the specificity, positive predictive value and negative predictive value There were no statistical significance in the sensitivity, specificity, positive predictive value, negative predictive value between IDUS + brush cytology + P53 and IDUS + brush cytology + K-ras [Table 3].

Endoscopic complications

All patients after endoscopic operation complicated by 2 cases of pancreatitis, 2 cases of biliary bleeding, 1 case of biliary infection and sepsis, were improved after conservative treatment, no perforation, and other complications, the complication rate was 5.95% (5/84).

Table 2: Comparison of the diagnosis value of single detection (% (*n*/*n*))

Items	IDUS	Brush cytology	K-ras	P53
Sensitivity	63.46 (33/52)	53.85 (28/52)	38.46 (20/52)[a1]	42.31 (22/52)[a2]
Specificity	78.13 (25/32)	100.00 (32/32)[b]	100.00 (32/32)[b]	100.00 (32/32)[b]
Positive predictive value	82.50 (33/40)	100.00 (28/28)[c]	100.00 (20/20)	100.00 (22/22)
Negative predictive value	56.82 (25/44)	57.14 (32/56)	50.00 (32/64)	51.61 (32/62)
Accuracy	69.05 (58/84)	71.43 (60/84)[d]	61.90 (52/84)	64.29 (54/84)

IDUS compared with the other three groups, $P<0.05$ ($\chi^2_{a1} = 6.50$, $\chi^2_{a2} = 4.67$, $\chi^2_{b} = 5.77$, $\chi^2_{c} = 3.73$); Brush cytology compared with K-ras mutation, $P<0.05$ ($\chi^2_{d} = 4.01$).

Table 3: Comparison of the diagnosis value of combined detection (% (*n*/*n*))

Items	IDUS + brush			
	Cytology	Cytology + K-ras	Cytology + P53	Cytology + K-ras + P53
Sensitivity	65.38 (34/52)[b1]	82.69 (43/52)[a2c1]	84.62 (44/52)[a1c2]	96.15 (50/52)
Specificity	84.38 (27/32)	90.63 (29/32)	90.63 (29/32)	93.75 (30/32)
Positive predictive value	87.18 (34/39)	94.38 (43/46)	93.62 (44/47)	96.15 (50/52)
Negative predictive value	60.00 (27/45)[b2]	76.32 (29/38)[a3]	78.38 (29/37)	93.75 (30/32)
Accuracy	72.62 (61/84)[b3]	85.71 (72/84)[a4c3]	86.90 (73/84)[c4]	95.24 (80/84)

IDUS + brush cytology + K-ras + P53 versus the other three groups: [a]$P<0.05$; [b]$P<0.01$. ($\chi^2_{a1} = 3.98$, $\chi^2_{a2} = 4.98$, $\chi^2_{a3} = 3.98$, $\chi^2_{a4} = 4.42$; $\chi^2_{b1} = 15.85$, $\chi^2_{b2} = 11.08$, $\chi^2_{b3} = 15.93$); IDUS + brush cytology versus IDUS + brush cytology + K-ras or IDUS + brush cytology + P53: [c]$P<0.05$ ($\chi^2_{c1} = 4.05$, $\chi^2_{c2} = 5.13$, $\chi^2_{c3} = 4.37$, $\chi^2_{c4} = 5.31$).

Surgery and pathology

Of 84 patients, 24 cases were performed operation, of which, 20 cases diagnosed as malignant biliary stricture by endoscopic IDUS and brush cytology (11 cases performed palliative surgery because of the metastases, 9 cases underwent radical resection due to no metastases), 4 cases found no tumor cell by brush cytology but suspected highly cancer by clinical follow-up (malignancy in 2 cases, inflammatory in 2 cases). Of all 22 cases with malignant surgical pathology, 19 cases were malignant adenocarcinoma (highly differentiated in 13 cases, low differentiated in 4 cases, undifferentiated in 2 cases), 2 cases were carcinoid, 1 case was squamous cell carcinoma.

Follow-up

All patients were followed-up for more than 12 months, average 14.6 months (range, 12.5–24 months). One, 3, 6, 12 month(s) after being discharged from our hospital, all patients came back for regular return visit. Blood biochemistry, blood tumor markers, MRCP, and CT were done to understand the process of benign and malignant disease. Of 52 cases with a final diagnosis of malignancy, nine patients were not found metastases in the other organs during the follow-up period 1-year after discharge, and achieved the purpose of early diagnosis, 43 cases with metastases (including 22 cases of surgical patients) showed obvious malignant manifestations during follow-up. No malignant syndrome occurred in 32 cases with benign biliary stricture during the follow-up period.

Discussion

Endoscopic retrograde cholangiopancreatography had been regarded as the "gold standard" in diagnosis of bile duct stenosis, but it was unsatisfactory that relying alone on ERCP to determine the nature of bile duct stricture that had the specificity of about 47% and accuracy of 73% according to the literature.[4] IDUS can scan the biliary and pancreatic duct using miniature ultrasound probe during ERCP procedure. Due to the advantage of being closer to lesions, less interference of abdominal fat and gas and higher image resolution, IDUS has become a new method performed to identify the nature of benign and malignant biliary strictures in recent years.[5-7] In this study, the sensitivity, specificity, positive predictive value, negative predictive value and accuracy of IDUS were 63.46%, 78.13%, 82.50%, 56.82% and 69.05% respectively in the diagnosis of malignant biliary stricture.

So far, pathology has been regarded as the "gold standard" in diagnosis of bile duct stricture. But a biopsy during ERCP has been limited in clinical application due to a long operating time, technical difficulty and a serious risk of biliary tract bleeding and perforation though it can theoretically get more and deeper diseased tissue. At the same time, the sensitivity of bile cytology was very low, the sensitivity of nasal aspiration cytology and bile duct drainage fluid cytology were respectively 30% and 24%.[8]

Fortunately, the value of brush cytology has gradually been reflected in the clinical.[9,10] It was previously reported that the sensitivity of brush cytology ranged from 18% to 60% and a specificity of approaching 100%.[6,11] Insufficient amount of cells was the main reason for the low sensitivity. The physician endoscopic operation techniques, experience of the pathologist, the patient's age, tumor size >1 cm and narrow length >1 cm were closely related to the positive rate of brush cytology.[12-14] In recent years, with more and more rich endoscopists operation experience, the sensitivity of brush cytology in the diagnosis of bile duct stricture was gradually improved, it was even more than 80% in some studies.[15-17] In this study, the sensitivity, specificity, positive

predictive value, negative predictive value and accuracy were respectively 53.85%, 100%, 100%, 57.14% and 71.43% in the diagnosis of malignant biliary stricture.

Currently, with the development of molecular biology techniques, the study of the incidence, development and biological behavior of malignant biliary stricture has entered the gene level.[18] So far, some genes were found to be associated with malignant biliary stricture include oncogenes ras (K-ras and N-ras), c-erbB-2 and c-myc gene; the tumor suppressor gene APC, DCC, and P53 and so on. Among them, the K-ras and P53 gene mutations were studied most widely and deeply.

Due to the low sensitivity and specificity of serum or bile K-ras, P53 gene mutations in detecting malignant biliary stricture, in recent years, brush cytology has been gradually used to detect K-ras and P53 gene mutations for the diagnosis of malignant bile duct stricture,[19,20] but the number of reported cases were less, and many of which were tested individual gene without the simultaneous detection of K-ras and P53 gene mutations.

To investigate the role of K-ras gene mutation in bile and brush cytology in the diagnosis of malignant bile duct stenosis, Wu *et al*.[21] detected K-ras gene mutation in 23 cases of bile specimens, 14 cases of bile duct brush cytology, 10 cases of bile duct carcinoma tissue by PCR-single strand conformation polymorphism and DNA sequencing. In the above study, the rate of K-ras gene mutation in bile, brush cytology and tissue was 54.5%, 60% and 60%, respectively. No K-ras gene mutation occurred in benign bile duct stenosis. The DNA sequence analysis showed point mutation in 2 cases, insertional mutagenesis in 4 cases. The rate and type of K-ras gene mutation in bile, brush cytology and tissue were consistent in the same case. So the authors believed that the detection of K-ras gene mutation in bile and brush cytology for diagnosis of bile duct cancer before surgery had largish practical value. To evaluate the diagnostic yield of brush cytology and the changes obtained by adding P53 and K-ras staining, Kim *et al*.[22] performed ERCP with brush cytology on 140 cases with biliary obstruction during a 7-year period. The sensitivity and specificity of brush cytology were 78.2–90.5%, respectively, the sensitivity of brush cytology + P53 was 88.2%, brush cytology + K-ras was 84.0%, and brush cytology + P53 and K-ras was 88.2%. The sensitivity of cytology + P53 was higher than that of brush cytology only (95% confidence interval: 83.69–92.78 vs. 72.65–83.65) but not that of cytology + K-ras.

In this present study, the sensitivity of K-ras and P53 was 38.46% and 42.31%, respectively, the specificity and positive predictive value were all 100% and the negative predictive value was 50.00% and 51.61%, respectively, accuracy was respectively 61.90% and 64.29%. The sensitivity of K-ras, P53 + brush cytology or IDUS was higher than that of K-ras or P53 in diagnostic malignant biliary stricture.

In short, IDUS has theoretically higher positive rate in the diagnosis of malignant biliary stenosis due to being performed ultrasound directly in the bile duct, closer to the lesion. But it's shortcomings is that it can't provide the pathological basis. After all, it belongs to the imaging diagnosis. The specificity of brush cytology in the diagnosis of malignant bile duct stenosis was 100%, but the sensitivity fluctuation large. In qualitative diagnosis of bile duct stenosis, single diagnostic measure is difficult to make a correct diagnosis, to combine several diagnostic techniques is helpful to make an accurate diagnosis. In this present study, the sensitivity and accuracy of K-ras and P53 + brush cytology and IDUS in the diagnosis malignant biliary stenosis were 96.15%, 95.24% respectively, significantly higher than that of any single detection or joint detection. And 9 cases had no metastasis when made a definite diagnosis, at the same time, no recurrence after being performed radical resection for 1-year. Therefore, IDUS combined with brush cytology and K-ras, P53 gene mutation detection contribute to the early diagnosis of malignant biliary stricture.

The limitation of this study is a single center study with small sample cases. A multi-center prospective study with larger samples needs to be performed imminently.

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