Biliary Ki-ras gene Mutation Analysis In Diagnosing Malignant Biliary Stricture

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Abstract

Background: Identifying the nature of biliary stricture whether benign or malignant is a prerequisite for subsequent management. The diagnosis of malignancy is difficult to be achieved with standard imaging or histopathological techniques in many situations. **Aim of the work**: is to evaluate the diagnostic value of detecting the Ki-ras gene mutation compared to bile fluid cytology and brush cytology to differentiate between benign and malignant strictures in a group of Egyptian patients. **Methods**: Ki-ras codon 12 point mutation was identified by PCR and RFLP in bile aspirate obtained during ERCP for 40 patients with biliary stricture (finally diagnosed as benign stricture in 15 patients and as malignant stricture in 25 patients) . Standard cytological examination was also performed on bile bile fluid and on brush cytology samples of the stricture. **Results**: Overall 13 patients (52%) in malignant group had mutant Ki-ras gene and 2 patients (13.3%) in benign group were positive for Ki-ras mutation. For diagnosing malignancy Ki ras gene mutation analysis had 52%sensitivity, 86.6% specificity while brush cytology had 8% sensitivity and 100% specificity. **Conclusion**: Ki-ras gene mutation analysis in bile had a higher sensitivity in making a diagnosis of malignant stricture when compared with either brush or bile fluid cytology. However the application of this molecular markers in clinical practice is limited by the costy procedure, the need to improve the sensitivity and specificity of this marker by the combination of several markers, use of quanitative analysis to define threshold values for malignancy diagnosis. [Nature and Science. 2009;7(5):19-28]. (ISSN: 1545-0740).

Introduction

Early and accurate diagnosis of pancreaticobiliary malignancy offers the best chance of a surgical cure while avoiding unnecessary major surgery in patients with benign disease. However, despite many advances in biochemical testing, tumour markers, non-invasive imaging techniques, endoscopic retrograde cholangiopancreatography (ERCP), and various tissue sampling techniques, the nature of a stricture may remain unclear **Khalid et al., 2004.**

Although cholangiographic features at endoscopic retrograde cholangiopancreatography (ERCP) may be characteristic for malignant or benign disease, the diagnosis of malignant biliary strictures still rests on the identification of tumor cells obtained using various methods, including open biopsy, ultrasound or computed tomography guided fine needle aspiration or core biopsy, endoscopic forceps biopsy, endoscopic brush cytology, and bile aspiration cytology **Fogel & Sherman, 1999 & Ponchon ,2000.** Biliary and pancreatic duct lesions are not always readily accessible to biopsy, and cytological techniques have become the initial diagnostic modality in many cases. The reported sensitivities of bile or pancreatic juice cytology or brush cytology are highly variable but remain relatively low ranging from 6% (bile duct) to 85% (pancreatic duct)**Van laethem et al., 1998.** Cytology performed directly on bile allows detection of malignant cells in

only 6-26% of cancer cases **Foutch, 1994.**

Since mutations involved in neoplastic progression may be able to serve as markers for the presence of small numbers of neoplastic cells that would otherwise escape detection in diagnostic assays **Dillon et al., 2000**; they can improve the sensitivity of standard histopathological evaluation. In this aspect Ki-ras codon 12 mutations represents one of the earliest genetic changes in the development of pancreatic cancer **Almoguera et al., 1988**. Such point mutations mainly reside in the first two nucleotides of codon 12, making their detection by polymerase chain reaction (PCR) feasible Amplification of DNA from bile specimens is also possible, revealing point mutations associated with malignant strictures of the main bile duct **Dillon et al., 2000.**

The frequency of Ki-ras codon 12 mutation in pancreatic cancer was estimated at 75% to 95% when investigating histopathological specimen **Trumper et al., 2002**. There remains debate as to the exact frequency in bile duct cancer **Motojima et al., 1991, Tsuda et al., 1992** and thus in malignant stricture as an entity caused by a variety of disorders **Stewart et al., 2001**. The potential use of this molecular technique in bile samples for confirming the presence or absence of malignancy in patients with biliary stricture generated much interest and enthusiasm

This study aimed to evaluate the diagnostic value of detecting the Ki-ras gene mutation in bile samples compared to bile fluid cytology and brush cytology to differentiate between benign and malignant biliary strictures in a group of Egyptian patients.

Patients and Methods

1. Patients

This study was conducted on 40 patients with obstructive jaundice due to biliary strictures, referred to the ERCP unit at the Department of Gastroenterology and Hepatology, Ain Shams University Hospital. For each patient a definite diagnosis of the nature of the stricture was established by histological confirmation in tissue samples during ERCP or subsequent surgery or by adequate follow up of at least six months by imaging methods as CT scan in combination with the clinical disease course. None of the patients diagnosed to have benign stricture showed evidence of a worsening status or the presence of a tumour mass at follow-up CT.

Group I**:** Twenty five patients with malignant biliary strictures. They were 15 patients with pancreatic adenocarcinoma, 8 patients with ampullary carcinoma, 1 with cholangiocarcinoma and 1 with gall bladder cancer.

Group II: Fifteen patients with benign biliary strictures. They were 3 patients with primary sclerosing cholangitis, 4 patients with postoperative biliary stricture and 8 patients with postinflammatory stricture associated with gallstones and chronic pancreatitis.

All patients enrolled in the study had to give their written consent after sufficient explanation of the research and procedures to be done.

2. Sampling technique/ ERCP

Under conscious sedation using midazolam 5 mg & pethidine 25-50 mg, ERCP was performed using Pentax EPM-3500. The scope is side-viewing so that the papilla can be seen "face-on" and cannulated with a 1.7 mm teflon catheter through a scope conduit.

Bile fluid (12 ml) was aspirated from the common bile duct using a 20 ml dry syringe applied to a Wilson-Cook catheter. 10 ml of he aspirated bile fluid was then flushed into a 15 ml sterile BD Vacutainer, and then transported to the histopathology laboratory within an hour for bile fluid cytology **Jin et al., 1999**.

Another 2 ml of bile was collected in sterile test tubes then centrifuged at 12000 rpm for 15 minutes in a cooler centrifuge (Beckman GS- 15R centrifuge). The pellet was washed twice with phosphate buffered saline and subsequently stored at -80 °C until used for DNA extraction **Sambrook & Russel, 2001**. Then filling of both the pancreatic duct and the bile duct system was attempted. The contrast medium used was Urovideo 75% (Amidotrizoic acid), introduced under low pressure by hand injection under fluoroscopic control until entire filling was reached for radiographic documentation

The following findings were assessed during cholangiography:

1 The level of the biliary stricture: proximal, middle or distal

2 The pattern of the stricture: smooth tapering or abrupt cut-off

3 The presence of shouldering

4 The presence of mucosal irregularity

5 The presence of filling defects

Then a wire guided cytology brush system (Wilson-Cook Medical, Inc., Winston-Salem, N.C.) was used. The brush was opened into the lumen above the stricture and moved back and forth across the stricture, approximately 10 to 15 times, in a to-and-fro fashion. The brush was pulled just into the tip of the catheter, and the catheter withdrawn. After the brushing was performed, the brush was smeared on 4 slides. The slides were fixed immediately in 95% alcohol.

If possible Malleable forceps (Olympus America, Inc., Melville, N.Y.) was used to obtain tissue from the distal rim of the stricture or from ampullary mass. 2-4 bites were taken and fixed in 10% formalin for standard histopathological examination.

3. Histopathological examination

Bile fluid cytology and brush cytology slides were stained by Papanicolaou stain. The cytopathological criteria for malignancy included nuclear enlargement, pleomorphism (minimum of 3-4 fold variation in nuclear size), elevated nuclear/ cytoplasmic ratio, nuclear membrane irregularity and coarse Chromatin, loss of honeycomb arrangement **Solcia et al., , 1997** , **Jin et al., 1999**. Those showing minimal features of the above criteria not satisfactory for malignancy diagnosis (low grade atypia) were not considered in sensitivity and specificity calculation of the results.

4. Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP) for Detection of Ki-ras Gene Point Mutation

A. DNA Preparation/Extraction:

Genomic DNA from the bile fluid pellets was extracted using the QIAamp DNA Mini Kit (QIAGEN Inc. Valencia, USA) according to manufacturer's instructions. To avoid DNA contamination, each of the samples was processed independently.

Measurements of DNA concentration were done by using Du series 640 spectrophotometer (Beckman Inc, USA).

B. DNA Amplification by Polymerase Chain Reaction (PCR):

The primers used (Metabion international AG, Deutschland) to detect the Ki-ras gene, were as follows:

- Primer (A) 5'-ACTGAATATAAACTTGTGGTAGTTGGACCT-3' (concentration: 100 Mm, molecular weight: 9260, number of bases: 30).
- Primer (B): 5'-TCAAAGAATGGTCCTGGACC-3' (concentration: 100 Mm, molecular weight: 6126, number of bases: 20)

- Primer (C): 5'-GCATATTAAAACAAGATTTAC-3' (concentration: 100 Mm, molecular weight: 6421, number of bases: 21)

To avoid false negatives, the presence of intact DNA was evaluated for each sample by control PCR using GAPDH. Sample that did not amplify with these primers were excluded from analysis. GAPDH was used as an internal control to monitor the integrity of the amplification process.

In a separate microfuge tube, the following components were prepared as a premix according to the number of samples, such that the total reaction volume was 50 µl.

Amplifications with Taq polymerase (Promega, Madison,W1) were performed in 100 µl reaction mixtures containing 2.5 units of Taq polymerase, 100 pmol of each primer, deoxyribonucleoside triphosphates (dATP, dCTP, dGTP, dTTP) at 200 µM each, 1.5 mmol/L MgCl2, 60 mmol/L KCl, and 10 mmol/L Tris-HCl (pH 8.8). The reaction mixtures were subjected to amplification using a Thermal cycler (Biometra, Uno II Thermoblock), each cycle comprising 94 °C for 1 minute, 55 °C for 1 minute, and 72 °C for 0.5 minutes. A negative (no DNA) control was run with each PCR analysis. The first PCR comprised 30 cycles followed by Mval digestion and a second PCR of 40 cycles.

C. Restriction Fragment Length Polymorphism Analysis (RFLP

Restriction enzyme digestion with Mval (Metabion international AG) was performed after each step of PCR in reaction buffer containing 16 µl of PCR product, 2 µl of the enzyme and 2 µl of the buffer. Mutation band was confirmed by electrophoresis of the second digested sample on 4% agarose gel and eluted.

PCR with primers A and B gave rise to a 157 base pair fragment containing two Mval restriction sites in case of normal codon 12, and just one site in case of presence of mutation in its first two bases. Therefore, wild type fragments cleaved to yield 114 base pairs, whereas mutant fragments yielded 143 base pairs.

The components used in polymerase chain reaction for K-*ras* gene detection were: Distilled water (30.75 µl), 10x reaction buffer (5µl) Primer 1 (sense) (5µl), Primer 2 (anti-sense) (5µl), dNTPs (10 Mm) (1µl), MgCl2 (25Mm) (3µl), Taq polymerase (5µ/µl) (0.25µl)

5. Statistical Analysis of the Results

Data were collected, revised then analyzed statistically using SPSS statistical package version 13. The following tests were applied: Mean value, Standard deviation, t- student test for independent sample means, Chi-square test

Sensitivity , Specificity , Positive predictive value (PPV) and Negative predictive value (NPV) were calculated as following: (Sensitivity = true positive/ true positive+ false negative, Specificity= true negative/ true negative+ false positive, Positive predictive value (PPV) = true positive/ true positive+ false positive & Negative predictive value (NPV) = true negative/ true negative+ false negative)

Significance was assumed when $P< 0.05$, high significance when $P<0.01$

Results

Ki-ras codon 12 analysis was successfully performed in 40 patients with obstructive jaundice due to biliary stricture [25 patients with malignant stricture and 15 patients with benign stricture], some demographic and laboratory data are shown in table (1) , Some ERCP findings are shown in table (2).

Table (1): Comparison between both studied groups as regard age, BMI, the serum liver enzymes and bilirubin.

variables	Benign n=15	Malignant n=25	t-value	p-value	significance
Age	45.8±17.9	60.2±10.1	3.257	<0.01	HS
BMI	26.4± 2.2	23.2± 1.6	-5.075	<0.01	HS
ALT (u/ml)	113.2□ 35.3	62.6 □28.5	-1.818	≥0.05	HS
AST (u/ml)	96.2 □71.6	90.1□ 51.2	-0.27	≥0.05	HS
ALP (u/ml)	230.8□ 130.4	493.8□ 206.2	4.424	<0.01	HS
γ-GT (u/ml)	117.0□ 90.9	239.0□ 123.0	3.329	<0.01	HS
Total bilirubin (mg/dl)	7.0□ 6.0	13.3□ 7.39	2.75	<0.01	HS
Direct bilrubin (mg/dl)	4.0□ 3.2	8.4□ 3.9	3.603	<0.01	HS

Table (2): Comparison between both studied groups as regard the radiological findings at ERCP.

sinificance	p-value	x2-value	Malignant	Benign	radiological findings
			n=25	n=15	
S	<0.05	4.235	6 (24.0%)	0 (0%)	Abrupt cut-off
S	<0.05	4.235	6 (24.0%)	0 (0%)	Mucosal irregularity
S	<0.05	4.952	12 (48.0%)	2 (13.3%)	Shouldering
H.S	<0.01	13.811	5 (20%)	12 (80.0%)	Smooth tapering

Overall 13 patients (52%) in malignant group had mutant Ki-ras gene and 2 patients (13.3%) in benign group (both with postinflammatory biliary stricture) were positive for Ki-ras mutation (table 3).
Distribution of Ki ras mutation in the malignant group is shown in table (4).

Table (3): Comparison between both studied groups as regard the presence of Ki-ras mutation by PCR.

Benign n=15	Malignant n=25	Ki-ras gene mutation
13 (86.7%)	12 (48.0%)	negative
2 (13.3%)	13 (52.0%)	positive
4.444	4.444	x^2 value
<0.05	<0.05	p value
Significant	Significant	Significance

Table (4): Distribution of Ki-ras mutation in malignant biliary strictures.

Malignancy	Ki-ras mutation		total
	negative	positive	n=25
Cancer pancreas	8 (53.3%)	7 (46.7%)	15
Ampullary cancer	2 (25%)	6 (75%)	8
Cholangiocarcinoma	1 (100.0%)	0 (0.0%)	1
Gall bladder cancer	1 (100.0%)	0 (0.0%)	1
x^2 value	0.746		
p value	≥0.05		
Significance	Not Significant		

Standard histopathological examination of bile fluid cytology and brush cytology was negative for malignancy in all patients in the benign group. In the malignant group only two patients were positive for malignancy in brush cytology, two patients show dysplasia described as few atypical cellular feature not satisfactory to diagnose malignancy (1 in bile fluid cytology & 1 in brush cytology) as shown in table 5

Table (5): Data of bile fluid cytology& brush cytology and Ki-ras mutation in the malignant group.

	Bile fluid cytology	Brush cytology	Ki-ras
	n=25	n=25	n=25
negative	24 (96%)	22 (88.0%)	12 (48.0%)
atypical	1 (4%)	1 (4%)	//
positive	0 (0%)	2 (8.0%)	13 (52.0%)
P value	<0.01		
significance	Highly significant		

For dignosis of malignancy sensitivity, specificity, PPV, NPV of bile fluid cytology, brush cytology and Ki ras gene mutation analysis are shown in table 6.

Table (6): Comparison between brush cytology and Ki-ras mutation in the malignant group as regards the sensitivity, specificity, positive predictive value and negative predictive values.

test	Bile fluid cytology	Brush cytology	Ki-ras mutation
sensitivity	0%	8.00%	52.00%
specificity	100%	100.00%	86.60%
PPV	0%	100.00%	86.60%
NPV	38.50%	40.50%	52.00%

Discussion

The diagnosis of malignant biliary strictures rests on the identification of tumor cells obtained using various methods; of which brush cytology performed at endoscopic retrograde cholangio-pancreatography (ERCP) has become a preferred method as it has a low complication rate and allows sampling from most sites within the pancreatic and biliary duct systems however it has modest sensitivity **Stewart et al., 2001**. It was hoped that the combination of standard diagnostic procedures & novel molecular tests designed to detect presumably tumour specific markers with a high sensitivity would greatly improved the diagnostic accuracy of biliary stricture **Trumper et al., 2002** . Ki-ras oncogene mutation is thought to be one of the earliest genetic changes in pancreatic cancer development **Almoguera et al., 1988.** It appears to be of biological significance in the complex process of cell transformation and has been described in several human carcinomas as well as in biliary tract cancers **Hruban et al, 1997**

In this study we compared the ability of Ki-ras codon 12 mutation analysis in bile fluid to diagnose malignancy compared to histopathological examination of brush cytology and bile fluid cytology.

Although brush cytology showed specificity of 100% for malignancy detection very low sensitivity (8%) was encountered. Data of previous studies are all in agreement concerning specificty of around 100%. The sensitivity rates reported previously are highly variable and range between 30% **Singh et al., 2003, Fogel et al.,2006** and 68% **Govil et al., 2002.** In a large series of 312 consecutive patients with extrahepatic bile duct stenosis the sensitivity of brush cytology for malignancy detection was not higher than 36% **Sturm et al., 1999**.

There are several possible explanations for the limited sensitivity of brush cytology in assessing pancreatic and biliary carcinomas. A low cellular yield is often the limiting factor in making a diagnosis of malignancy; in a recent study increasing brush size and bristle stiffness does not increase detection rates **Fogel et al., 2006**. In addition sampling errors might occur when tumours at these sites show a predominantly submucosal spread, with limited or absent surface epithelial abnormality. **Kurzawinski et al., 1993** & **Foutch, 1994** Similarly, strictures might be caused by external compression without directly involving the ductal epithelium that is why in general, results of brush cytology for biliary strictures induced by pancreatic malignancies have proved to be inferior (on average 46%) to those observed for biliary malignancies (on average 68%). **Glasbrenneret al., 1999**.

In addition some of these wide variations in sensitivity of brush cytology in reports arise from inconsistencies in the criteria used for classification of cells on cytological slides. These inconsistencies mainly arise in the categorization of lesions not fulfilling all criteria of malignancy. Classification of such cells has been termed to be a "cytological grey zone" and includes categories such as atypical, dysplasia (low and high grade), and suspicious **Selvaggi, 2004**. The morphological criteria used for this classification show significant overlap in various studies, and some authors even include suspicious or atypical lesions in the calculation of sensitivity. Thus comparison of the sensitivity and specificity rates obtained in various studies are hampered by the inconsistencies in the definition of cytological criteria in this "cytological grey zone" **Gress, 2004**

Concerning bile fluid cytology, all patients with benign strictures and 96% of those with malignant disease had a normal bile fluid cytology. Only one patient (4%) with malignancy had low grade atypia. It is reported in many studies that bile cytology is less sensitive than brush cytology **Kurzawinski et al.,1993 & Savader et al., 1998.** as in addition to the previously described factors limiting sensitivity of cytological examination of biliary malignancy in general, for bile fluid cytology a large volume of bile fluid may be needed to yield a few malignant cells.

In the present study Ki-ras gene mutation had a sensitivity of 52% and a specificity of 86.6% in diagnosing malignancy. This means that, according to our results, Ki-ras mutation had a higher sensitivity but a lower specificity in diagnosing a malignant biliary stricture.

A study by **Dillon et al., 2000** revealed that Ki-ras gene mutation had a sensitivity and specificity of

33% and 79% respectively. In the study performed by **Saurin et al., 2000**, the reported sensitivity, specificity and positive predictive values of Ki-ras mutation analysis in bile fluid samples of cases with malignant strictures were 17%, 96% and 94% respectively. Also, **Trumper et al., 2002** showed that Ki-ras mutation had a relatively low sensitivity of 38.1% but a high specificity of 90.5%. On the other hand **Van Laethem et al., 1998** compared Ki-ras mutation with brush cytology in 142 patients with malignant biliary strictures. The study reported a sensitivity of 25% versus 42% and a specificity of 100% versus 100% for Ki-ras mutation and brush cytology respectively.

The differences between the studies as regards the prevalence of Ki-ras mutations in malignant biliary strictures could be attributed to the different proportions of patients with malignancy enrolled in each study with different prevelance of the mutation which is reported in the literature to vary widely between 0% and 100 % **Gress, 2004**. According to **Saurin et al., 2000**, the prevalence of Ki-ras mutation in tissue samples obtained directly from the tumour was as follows: 90% in pancreatic adenocarcinoma, 77% in cholangiocarcinoma and 55% in gall bladder cancer and all these values are certainly higher than that detected in bile fluid cytology or brush cytology. The location of the biliary tumour (proximal or distal bile duct, intrahepatic bile ducts, gall bladder), racial and geographic variation, as well as the methods used for mutation detection have been assumed to cause these differences in the incidence of K-ras codon 12 mutations **Gress, 2004**.

The sensitivity of Ki-*ras* mutation analysis is however limited by the prevalence of these mutations in the tumour type under investigation, and is dependent on the presence of a sufficient amount of tumour cells in the biological sample used for the analysis. The specificity of Ki-ras PCR analysis is limited by the possible occurance of the mutation in non- malignant diseases of the pancreatic duct. **Furuya et al., 1997** showed that up to 37% of patients with chronic pancreatits showed mutation of the Ki-ras gene.

According to our study, the Ki-ras gene mutation was detected in the bile samples in 6 out of 8 (75%) cases of ampullary cancer. Contrary to our findings, **Ito et al., 1998 & Motojima et al., 1991** postulated that Ki-ras analysis was useful in differentiating true pancreatic from ampullary malignancy which are clinically likely to be confused in several cases as the later has no Ki-ras mutation. Although the number of ampullary cancer patients in our study was small, our findings at least highlighting the inappropriate use of Ki-ras mutation in this aspect in Egyptian patients till more data are available.

In conclusion, our study showed that Ki-ras gene mutation analysis in bile had a higher sensitivity in making a diagnosis of malignant stricture when compared with either brush or bile fluid cytology. However the application of this molecular markers in clinical practice is limited by the costy procedure, the need to improve the sensitivity and specificity of this marker by the combination of several markers, use of quanitative analysis to define threshold values for malignancy diagnosis as number of mutant cells will be much lower in inflammatory than in malignant cells.

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