

p53 and DPC4 Alterations in the Bile of Patients With Pancreatic Carcinoma

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Introduction: Pancreatic cancer is still predominantly diagnosed in advanced stages, and 85%–90% of patients are not eligible for surgery at diagnosis. This is mainly due to the great difficulty in detecting the tumour at an early stage and presently no satisfactory results have been obtained to overcome this problem. Studies on molecular genetic profile of pancreatic cancer may represent an important approach. This study was focused on the mutations of p53 and DPC4 detectable in the bile of patients with histologically proven pancreatic cancers.

Materials and Patients: We analysed specimens of bile collected through percutaneous transhepatic biliary catheters, placed to treat malignant biliary obstruction in 25 patients with pancreatic adenocarcinoma. A percentage of mutation was obtained of 43 % for the microsatellite D17S945 (p53), 54% and 50 % for D18S46 and D18S474 (DPC4), respectively. The percentage of amplification was 67%, 93,6%, and 80%.

Conclusion: We consider the results encouraging enough to decide to enlarge the number of patients examined. The aim is to determine if a test for DPC4 and p53 mutations is eligible for introduction in clinical routine use. More sets of samples are required to satisfactorily answer this question.

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KEY WORDS: microsatellites; genetic alterations; pancreatic neoplasms

INTRODUCTION

Pancreatic cancer is still predominantly diagnosed in advanced stages of the disease, and 85%–90% [1–6] of patients are not eligible for surgery at diagnosis. This is mainly due to the great difficulty in detecting the tumour while it is still at an early stage and despite the various strategies available at the present moment (i.e., tumour markers, new imaging techniques) no satisfactory results have been obtained to overcome this problem.

Studies about tumour biology and molecular genetic profile of pancreatic cancer initiation and progression are numerous in literature. The main aim is to obtain a better knowledge about the process of tumorigenesis of pan-

creatic cancer, and the disease's relation to morphological features and prognosis [7–14].

Pancreatic cancer is known to be the result of accumulated genetic modifications. The progression appears

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TABLE I. Summary of Genetic Alterations in Sporadic Pancreatic Adenocarcinomas

Gene	Frequency of mutations (%)	Gene location
Oncogene		
k-ras	95	12p12
Tumor suppressor gene		
p-16	>90	9q21/13q14
p-53	50–75	17p31
DPC4	55	18q21q

to be conform to the Vogelstein model, as described for colorectal cancer [15]. The most common alterations are subtle sequence changes in the oncogenes and mutations and deletions of tumour suppressor genes [16–24]. Among the genetic somatic alterations under investigation, the oncogene *k-ras* and the tumour suppressor genes, *p53*, *p16*, and *DPC4* mutations have also been found to play a critical role in the progression of pancreatic cancer (see Table I) [25–36]. These alterations appear in a sequence of progression that culminates in the emergence of pancreatic ductal cancer [18,37,38]. This pattern has been defined by an international expert committee that classified the lesions on the basis of histological criteria in three grades of pancreatic intraepithelial neoplasia, (http://pathology.jhu/pancreas_panin) [39,40] and also defined their progression towards the final complete appearance of the malignant pancreatic neoplasia.

The late appearance of *p53* and *DPC4* mutations and the relationship between *p53* and *k-ras* mutation could represent additional criteria for the identification of the anaplastic lesion PANin stage 3, the late step of the tumorigenesis before the appearance of an invasive adenocarcinoma [39–41]. The mutations of the *k-ras* gene, found in 90% of pancreatic cancer, have also been identified in numerous benign conditions, including chronic pancreatitis [42–44]. For the latter characteristic, we prefer to consider *p53* and *DPC4*. In fact, *p53* has an association with *k-ras* mutation suggesting a cooperative effect in tumorigenesis [41,42,45,46]. The abnormal expression of the *p53* protein is present in genetic profile of human carcinomas and it is inactivated in 40%–75% of pancreatic cancer [10,42]. Its alteration is frequently seen in PANin stage 3. Primary mutation mechanism is homozygous deletion, and it plays a role in many regulatory pathways. The protein expressed by *p53* responds to cellular DNA damage and may block the cell progression through the G1 phase of the cell cycle.

DPC4 mutations were frequently seen in PANin 3. This tumour suppressor gene is deleted in approximately 50% of pancreatic cancer, and up to 90% of pancreatic carcinomas may harbour loss of heterozygosity [28,42]. The product of the gene is a component of the TGF- β signal

transduction pathway, probably involved in a more invasive growth pattern [42,46,47].

Since it is known that tumour DNA have been isolated from a variety of sources (blood, pancreatic fluid, stool, bile, urine) and the detection of cancer derived gene products is possible from biologic fluids [47–54], the identification of genetic abnormalities of *p53* and *DPC4* could be rightly considered an important emerging approach in diagnosis. In our study, we focus on the mutations detectable in the bile of patients with histologically proven pancreatic cancers. The aim of the research is to evaluate if the detection of the mutations in *DPC4* and *p53* may be a valuable test for application in clinical practice. Our opinion is that the thus selected genetic markers, which are altered in the final stage of tumorigenesis (PANin stage 3), may represent a reliable starting point to possible clinical application.

MATERIALS AND METHODS

Patients

We studied 25 patients affected by pancreatic carcinoma, determined histologically. Specimens of bile were obtained by aspiration directly from percutaneous transhepatic biliary catheters previously placed to treat malignant biliary obstruction. The catheter traversed the hepatic parenchyma, and terminated in the common bile duct or in the proximal duodenum. From each patient 20 ml of bile was collected and a blood sample for the cariotype control. When possible, we tested also the frozen sections and paraffin embedded specimens of the tumour (i.e., fine needle biopsy or surgical resected specimens) of these patients.

Methods reliability was also evaluated [56]. Bile samples were collected from 10 patients without history of gastrointestinal (g.i.) cancer, through the suction tube positioned into the duodenum during open surgery for abdominal aortic aneurysm under viewing control by the vascular surgeons of our Institute. The DNA extraction method was also tested from duodenal fluid and bile.

In addition, we also evaluated the genetic alteration in the paraffin embedded specimens of the other 10 patients with a histologically proven pancreatic cancer, followed by us after surgery, and also paraffin embedded specimens of 5 patients who underwent surgery for chronic pancreatitis.

GENETIC ALTERATIONS

According to the recent genetic pathway proposed by Hruban et al. [39], we focused our study on the genes reported in Table I, in particular two tumour suppressor genes were selected whose mutation represent the final step toward the complete appearance of a pancreatic cancer: *p53* and *DPC4*.

The rationale for the study of genetic alteration in physiologic fluids, is referred widely in literature [47–54].

To extract DNA from the bile specimens, we used a method similar to which previously described by Abruzzese and colleagues [52]: each bile specimen was centrifugated at room temperature for 10 min at 1,550 rpm in a bench-top centrifuge, and the cellular contents were separated from the supernatant and frozen. DNA was then extracted.

Each frozen pellet specimen was mixed with 100 µl of 10 mg/ml proteinase K and incubated at 37°C for 1 hr; then it was thawed and mixed with an equal volume of phenol saturated with 10 mM Tris and 1 mM EDTA, pH 7.4. The mixture was centrifuged for 10 min at 2,000 rpm. The supernatant was then recovered and precipitated with absolute alcohol. The precipitated DNA was suspended in deionised water. The DNA recovered was then quantified spectrophotometrically.

The DNA was extracted from the specimens embedded in formalin, after xylene washes out (37°C) via the above-mentioned method [55,56].

The analysis of microsatellites was performed as referred by F. Canzian et al. in 1996 for the detection of LOH and RER [57].

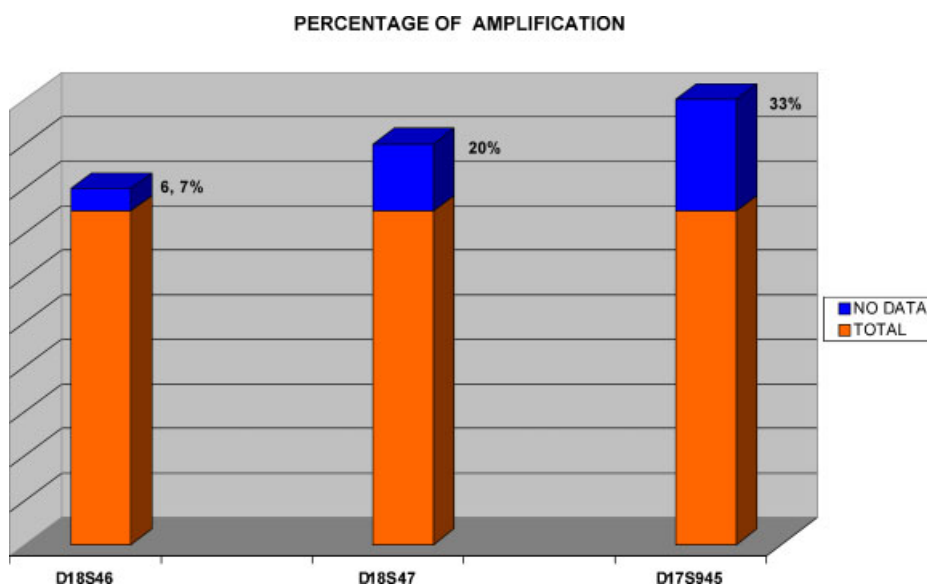
Microsatellites were studied according to the following procedure [55–57]: three markers to study loci from different parts of the genome and representing regions of potential interest in pancreatic cancer were expressly chosen [58] for tumour suppressor genes. The oligonucleotide D17S945F (AACCAATCTGG-

ACTCCCC), D17S945R (CCTGAAGCCTGACCC-CC), D1846F (GAATAGCAGGACCTATCAAAGAGC), D18S46R (CAGATTAAGTGAAAACAGCATATGTG), D18S4774F (TGGGGTGTTTACCAGCATC), and D18S474R (TGGCTTTCAATGTCAGAAGG) were labelled fluorescently with one of three dyes (6-FAM, TET, HEX: Applied Biosystem, Forster City, CA) and the fourth dye (TAMRA) was reserved for the size standard (Table II).

The PCR reactions for fluorescent markers were performed in a volume of 25 µl and included 1× PCR buffer, 1.5 mM MgCl₂, 250 µM of each dNTP, 30 ng DNA, 0.6 U Dynazyme Taq Gold polymerase (Applied Biosystems, Norwalk, CT), and 3 pmol of each primer (one of them fluorescently labelled). The thermal profile was as follows: 95°C for 13 min; 30 cycles of 95°C for 45 sec, 55°C for 30 sec, and 72°C for 45 sec and a final extension of 72°C for 45 min.

The three fluorescent products for each template were mixed for electrophoresis and allele scoring (fluorescent techniques) each consisting of 15 µl. Two micro-litres of this mixture were added to 3 µl formamide and 0.5 µl TAMRA 500 size standard (Applied Biosystems), loaded on a 6% polyacrilamide 8M urea gel, and run 8 hr in a 373 A ABI PRISM Automated Sequencer (Applied Biosystems). For data automatic collection and analysis the Gene Scan software (Applied Biosystems) was used, while for the allele scoring and assessment of LOH and RER+ were employed the Genotype software (Applied Biosystems) and the Excel software (Microsoft, Redmond, WA) were used [59].

TABLE II. Rate of Amplification: The Total Number of Analyzed Samples is Represented in Red and the Percentage of Noninformative Data is Represented in Blue. The Percentage of Amplification was 67% (p53, Microsatellite D17S945), 93.6% (DPC4, Microsatellite D18S46) and 80% (DPC4, Microsatellite D18S474)



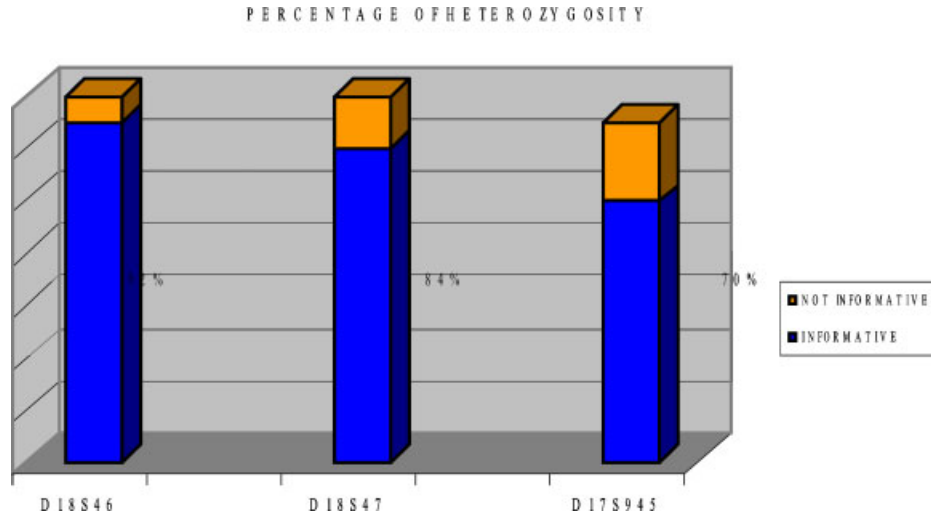


Fig. 1. The percentage of heterozygosity for the microsatellite markers D18S46 and D1847 (DPC4) and D17S945 is represented in blue: it was 92%, 84%, and 70% respectively. The noninformative percentage is represented in red.

AUTOMATED CALCULATION OF LOH AND RER FOR SIMPLE GENOTYPE

The genotype labels the alleles of the normal tissue and the corresponding peaks in the tumour tissue, then all the detectable peaks. The data are then passed to Excel for additional calculation [59].

RESULTS

Results obtained from the bile and duodenal fluid examination showed a percentage of mutation of 43% for

the microsatellite D17S945 (p53), 54%, and 50% for D18S46 and D18S474 (DPC4), respectively. The rate of amplification is depicted in Table II: the percentage of amplification was 67% (p53, microsatellite D17S945), 93.6% (DPC4, microsatellite D18S46), and 80% (DPC4, microsatellite D18S474). The results of amplifications are informative in respect to literature. The percentage of heterozygosity for the microsatellites markers D18S46, D1847 (DPC4), and D17S945 is depicted in Figure 1: the percentage of heterozygosity, it was 92%, 84%, and 70%, respectively. The results (percentage) of the samples

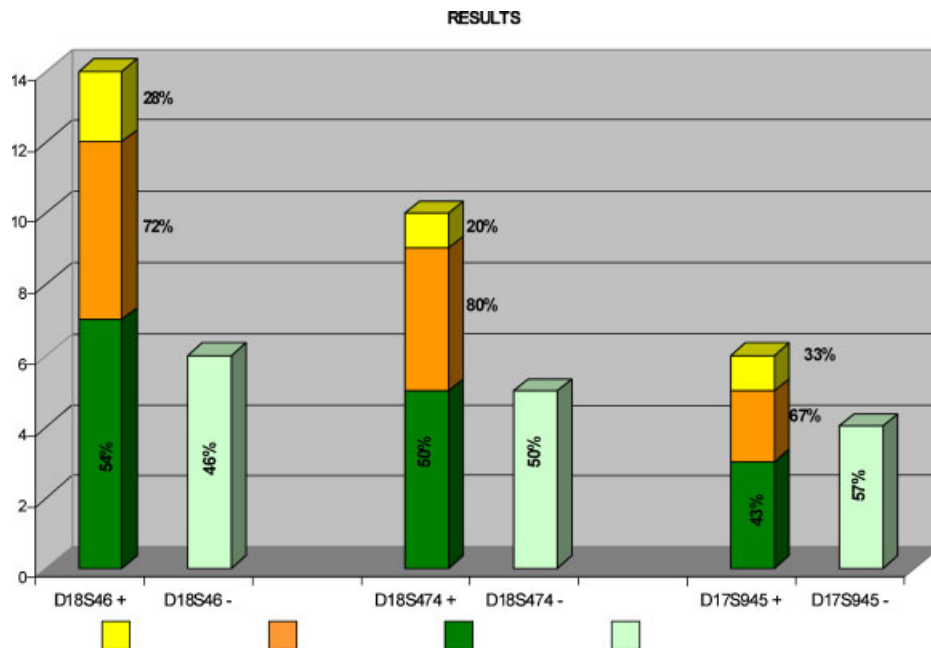


Fig. 2. The results (percentage) of the samples' heterozygosity are compared and summarized: the total number (represented in percentage) of alterations are depicted in dark green (54% for D18S46, 50% for D18S474, 43% for D17S945), the percentages of RER+ and LOH are represented in yellow and red respectively for each microsatellite (LOH: 72% and RER+: 26% for D18S46, LOH: 80% and RER+: 20% for D18S474, LOH: 67% and RER+: 33% for D17S945). The negative samples (percentage) are depicted in light green.

heterozygosity are compared and summarised in Figure 2: the total number (represented in percentage) of alterations are 54% for D18S46, 50% for D18S474, and 43% for D17S945, the percentage of RER+ and LOH are: LOH: 72% and RER+: 26% for D18S46, LOH: 80% and RER+: 20% for D18S474, LOH: 67% and RER+: 33% for D17S945. The negative samples (percentage) are also represented: 46% for D18S46, 50% for D18S474, and 57% for D17S945.

An analysis example of LOH and RER+ was reported in Figures 3 and 4.

No genetic alteration was found in the aspirate specimens of the control group. None of the genetic alterations described above, were present in the paraffin embedded specimens of chronic pancreatitis group.

DISCUSSION

Pancreatic cancer is caused by mutations in some cancer related genes [39,40]. Knowledge of these mutations conducts to the evaluation of their clinical impact.

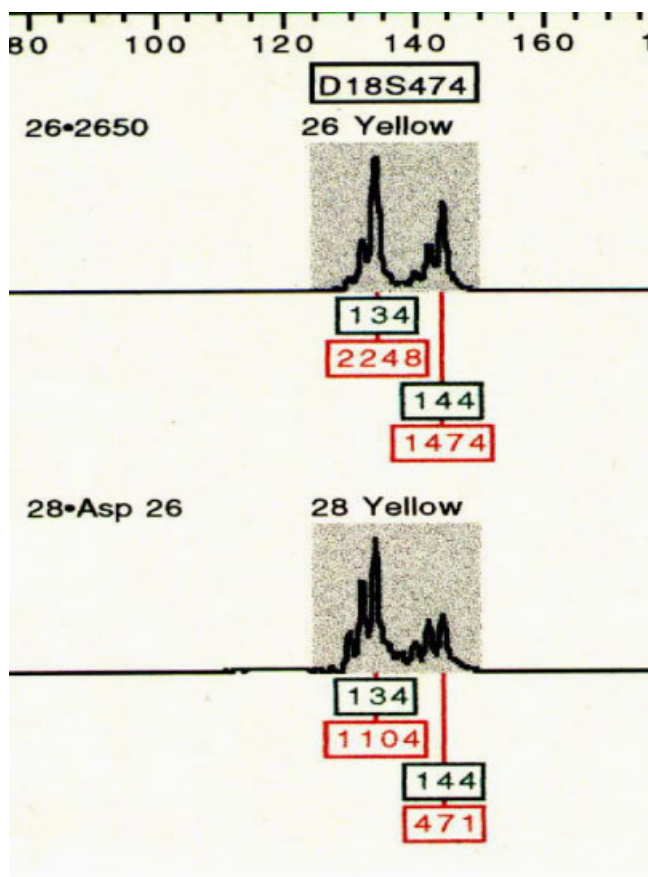


Fig. 3. Example of LOH.

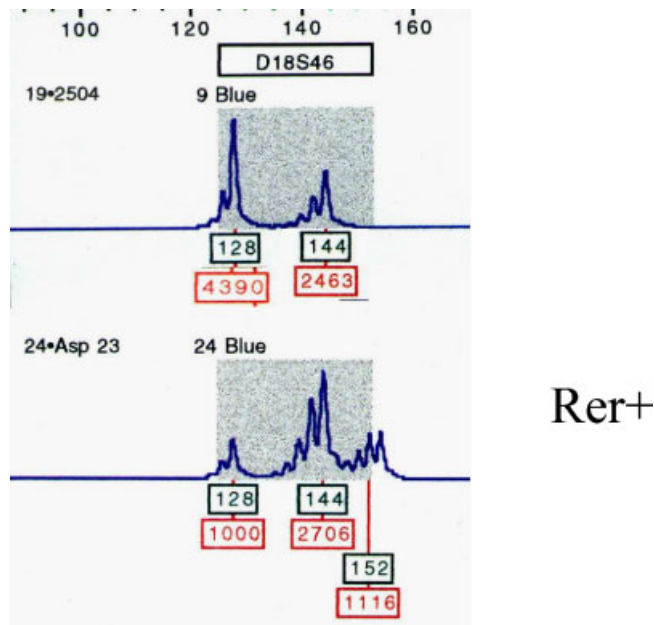


Fig. 4. Example of RER+.

In this study, we examined two tumour suppressor genes that are present mainly in the final step towards complete pancreatic cancer transformation (PANin stage 3): DPC4 and p53.

We used samples of bile and duodenal fluid of patients affected by pancreatic cancer. In the literature these sources are used for genetic examination [52,53], mainly for k-ras analysis. According to recent findings on the progression of the malignant transformation [39], we decided to test p53 and DPC4 due to their appearance in cancer progression corresponding to PANin stage 3.

These genes are inactivated by different mechanism (homozygous deletions HD, loss of heterozygosity-LOH, hypermethylation of the genes' promoter).

P53 resides on chromosome 17p and is inactivated in 50%–75% of pancreatic cancers. The protein products of p53 gene activate the transcription of a large number of genes involved in the apoptosis process, causing G1/S1 cell cycle arrest and stabilising the G2/M cell cycle checkpoint [8,11,33–40,58,60].

DPC4 resides on chromosome 18q [16,28,31], and the protein products of this gene are probably involved in signal transduction from the transforming growth factor (TGF- β) surface receptors. The phosphorylation of DPC4 leads to an activated complex that gets translocated to the nucleus, where it binds to DNA stimulating the transcription of nearby genes. It is generally known that DPC4 gene is inactivated in about 55% of pancreatic carcinoma and that alterations are by HD and LOH. It is also important to point out that DPC4 gene is relatively specific for pancreatic cancer and less commonly targeted in other tumour types [33,54].

This study is particularly focused on the possibility to use genetic abnormalities for differential diagnosis and screening procedures.

It is, in fact, a well-known problem that other pancreatic diseases, like chronic pancreatitis, can hardly be differentiated from a pancreatic cancer even under histopathological examination. The presence of mutated DPC4 and p53 could be associated with the presence of malignant neoplasia instead of a chronic pancreatitis [49]. We presently do not have bile specimens of histologically proven chronic pancreatitis, and we performed the analysis on paraffin embedded specimens of patients surgically treated, to compare with the paraffin embedded specimens of patients that underwent surgery for pancreatic cancer. No evidence of the genetic alterations reported in this study was detectable in the paraffin embedded specimens of the chronic pancreatitis group.

In the literature it is widely reported that genetic alterations provide new targets for developing new tests for screening purposes [47–49,51,61–66].

The results obtained from the bile and duodenal fluid of our patients showed a percentage of mutation of 43% for the microsatellite D17S945 (p53), 54% for D18S46, and 50% for D18S474 (DPC4). The percentage of amplification was 67%, 93.6%, and 80%, respectively.

CONCLUSION

All our data suggest to enlarge the number of examined patients, in particular to confirm the results on chronic pancreatitis versus pancreatic cancer present in the paraffin embedded set of samples, on bile specimens or frozen specimens also. Furthermore, since results for DPC4 correspond to those found in the literature, whereas the p53 values were lower, we think it would be opportune to extend the study, enrolling more patients.

We think a larger set of samples might clarify if the DPC4 and p53 alterations detectable in bile could be usefully employed in clinical practice for differential diagnosis and screening purpose.

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