Pancreatic tumours: molecular pathways implicated in ductal cancer are involved in ampullary but not in exocrine non ductal or endocrine tumorigenesis

PS Moore1, S Orlandini1, G Zamboni1, P Capelli1, G Rigaud1, M Falconi2, C Bassi2, NR Lemoine3 and A Scarpa1

1Department of Pathology and 2Department of Surgery, Università di Verona, Italy; 3Imperial Cancer Research Fund Molecular Oncology Unit, Imperial College School of Medicine, London, UK

Summary Alterations of K-ras, p53, p16 and DPC4/Smad4 characterize pancreatic ductal cancer (PDC). Reports of inactivation of these latter two genes in pancreatic endocrine tumours (PET) suggest that common molecular pathways are involved in the tumorigenesis of pancreatic exocrine and endocrine epithelia. We characterized 112 primary pancreatic tumours for alterations in p16 and DPC4 and immunohistochemical expression of DPC4. The cases included 34 PDC, 10 intraductal papillary-mucinous tumours (IPMT), 6 acinar carcinomas (PAC), 5 solid-pseudopapillary tumours (SPT), 16 ampulla of Vater cancers (AVC) and 41 PET. All tumours were also presently or previously analysed for K-ras and p53 mutations and allelic loss at chromosomal arms 9p, 17p and 18q. Alterations in K-ras, p53, p16 and DPC4 were found in 82%, 53%, 38% and 9% of PDC, respectively and in 47%, 60%, 25% and 6% of AVC. Alterations in these genes were virtually absent in PET, PAC or SPT, while in IPMT only K-ras mutations were present (30%). Positive immunostaining confirmed the absence of DPC4 alterations in all IPMT, SPT, PAC and PET, while 47% of PDC and 38% of AVC were immunonegative. These data suggest that pancreatic exocrine and endocrine tumorigenesis involves different genetic targets and that among exocrine pancreatic neoplasms, only ductal and ampullary cancers share common molecular events. © 2001 Cancer Research Campaign http://www.bjcancer.com

Keywords: pancreas; carcinoma; intraductal papillary-mucinous tumour; acinar cancer; solid pseudopapillary tumour; ampulla of Vater cancer; endocrine tumour; K-ras; p16; p53; DPC4/Smad4; microsatellites; allelotyping

Common pancreatic ductal adenocarcinoma (PDC) is characterized by a relatively unique molecular fingerprint constituted by frequent alterations of the K-ras (Almoguera et al, 1988; Lemoine et al, 1992;), p53 (Barton et al, 1991; Kalthoff et al, 1993; Scarpa et al, 1993a), p16\(^{INK4a}\) (Caldas et al, 1994) and DPC4/Smad4 genes (Hahn et al, 1996). Numerous studies on K-ras and p53 have confirmed that they are mutated at high frequency in primary PDC (K-ras reviewed in Hruban et al, 1993 and Scarpa et al, 1994a; for p53 see: Pellegrata et al, 1994; Redston et al, 1994). Less information is available regarding alterations of p16 and DPC4 in primary PDC and the only two available reports suggested that alterations in these two genes occur less frequently in primary PDC than in derived cell lines (Huang et al, 1996; Bartsch et al, 1999a). Studies on xenografts and cell lines have shown that homozygous deletions may account for up to half of all instances of inactivation of these genes (Caldas et al, 1994; Hahn et al, 1996; Naumann et al, 1996; Villanueva et al, 1998) although this phenomenon is difficult to demonstrate in primary PDC due to the high admixture of nonneoplastic cells in these tissues. In addition, the p16 gene may also be silenced by promoter methylation (Schutte et al, 1997), an epigenetic event not yet examined in a series of primary PDC.

Very little is known about the molecular abnormalities in neoplasms other than ductal arising from the exocrine pancreas, including intraductal papillary-mucinous tumours (IPMT), solid-pseudopapillary tumours (SPT), the cystic group of malignancies, the extremely rare acinar cell carcinomas (PAC), and cancers arising from the ampulla (papilla) of Vater (AVC) (Klöppel et al, 1996; Solcia et al, 1997).

Based on the mutational analysis of the K-ras and p53 genes in 35 PDC, 6 pancreatic exocrine non ductal and 12 pancreatic endocrine tumours (PET), an earlier study concluded that the molecular pathogenesis of exocrine non ductal and endocrine tumours involves pathways different from those involved in PDC (Pellegrata et al, 1994). However, two recent reports of highly frequent alterations of the p16 and DPC4 genes in PET suggested that common molecular pathways are involved in the tumorigenesis of exocrine and endocrine epithelia (Muscarella et al, 1998; Bartsch et al, 1999b).

To address these issues specifically, we have analysed 112 primary pancreatic tumours of different types, including 34 PDC, 37 exocrine non ductal and 41 PET, for molecular alterations in p16 and DPC4 genes. The inactivation of the DPC4 gene by additional mechanisms such as homozygous deletion was studied using immunohistochemistry, which has been shown to correlate with inactivation of the gene (Wilentz et al, 2000). All tumours were also presently or previously analysed for K-ras and p53 mutations and allelic loss at chromosomal arms 9p, 17p and 18q at sites linked to DPC4 genes. This study represents the largest report to date on the mutational status of different pancreatic tumour types for the 4 genes most commonly altered in pancreatic ductal cancers and provides evidence that only ductal and ampulla of Vater cancers share common molecular anomalies, while the less common tumour entities have a distinct molecular pathogenesis.
MATERIALS AND METHODS

All the studies performed were approved by the Ethics Committee of Verona University.

Primary tumours

The 112 frozen pancreatic tumours consisted of 34 PDC, 37 exocrine nonductal and 41 PET. The nonductal exocrine tumours were composed of 10 IPMT, 6 PAC, 5 SPT and 16 AVC. The PETs included 30 nonfunctional and 11 functional tumours. A neoplastic cellularity of at least 60% for PDC and ranging from 70% to virtually 100% for all other tumour types was obtained in all cases by either cryostat enrichment or microdissection of the frozen tumour samples, as described (Achille et al, 1996a; Soria et al, 1999).

Among the 34 PDC, 18 cases had been previously analysed for mutations in the K-ras and p53 genes; in particular, PDC3-PDC6 and PDC9-PDC11 were described in Achille et al (Achille et al, 1996a) and PDC19-PDC29 correspond to previously reported cases 1, 4, 5, 7, 8, 10, 11, 12, 14, 15 and 21 (Scarpa et al, 1993a). The remaining 16 cases have not been previously reported.

The 10 IPMT were characterized by diffuse or segmental dilatation of the main and/or branch pancreatic ducts with intraductal growth pattern sometimes forming intraluminal masses, which presented a wide spectrum of modifications ranging from low-to high-grade dysplasia and from carcinoma in situ to invasive cancer (Klöppel et al, 1996; Solcia et al, 1997). In particular, 3 cases had only the intraductal component and were considered of borderline malignancy, according to established criteria (Klöppel et al, 1996; Solcia et al, 1997). 7 cases also had an invasive component which was represented by mucinous carcinoma in 6 and ductal-like cancer in one. Cases IPMT1-IPMT4 correspond to cases 4, 5, 2 and 3, respectively, of a previous report in which they had been analysed for K-ras and p53 mutations (Sessa et al, 1994).

The 5 SPT were from prepubertal girls or young women, and characteristically showed progesterone receptor immunostaining (Zamboni et al, 1993). These cases have not been previously reported.

The 6 PAC were diagnosed by histopathological criteria and cell marker analysis. The latter confirmed the acinar nature of the neoplastic cells, which expressed lipase in all cases, amylase in 2 cases, trypsin in 5 and chymotrypsin in 4 cases (Klimstra et al, 1992; Hoorens et al, 1993; Solcia et al, 1997). These cases have not been previously reported.

The 16 AVC were selected by applying strict topographical criteria obtained at gross and histological examinations. Only small lesions with unequivocal ampullary origin, topographically centred in the region of the papilla of Vater were included in the study (Achille et al, 1996b, 1998; Scarpa et al, 1993b, 1994b). AVC showing microsatellite instability of the type seen in replication error phenotype (RER+) cancers were excluded from the study (Achille et al, 1997). All cases except AVC16 have been previously described and analysed for mutations in the K-ras and p53 genes and for 17p and 18q LOH (Scarpa et al, 2000).

The series of 41 PET was composed of 30 nonfunctional and 11 functional tumours, including 9 insulinomas, 1 gastrinoma and 1 VIPoma. 23 tumours were benign and 18 malignant, in accordance with the respective absence or presence of invasion of the neighbouring organs and/or nodal/distant metastases, as evaluated by imaging techniques, surgical and pathological examinations. All tumours were characterized using a panel of monoclonal antibodies recognizing pan-endocrine markers (chromogranin A, synaptophysin and non-specific enolase) and gastrointestinal hormones (insulin, glucagon, somatostatin, pancreatic polypeptide, gastrin, serotonin, and vasoactive intestinal peptide). Only those tumours giving rise to an endocrine syndrome were considered as functional. PETs were considered nonfunctional if clinical symptoms were absent, regardless of the immunostaining results. Among the 30 nonfunctional tumours, 24 showed no immunoreactivity for the tested hormones, while 4 tested positive for glucagon and 2 for somatostatin. Mutations in the K-ras and p53 genes for tumours NF1–NF10 and F1–F6 have been previously reported (Beghelli et al, 1998). All other cases have not been previously reported.

Loss of heterozygosity analysis

Most cases were analysed for allelic loss with high-molecular weight DNA, although some analyses were performed with DNA extracted from paraffin-embedded tissues prepared as described (Scarpa et al, 2000). Two microsatellites each were used for chromosomal arms 9p, 17p, and 18q. All cases were analysed for allelic loss using the microsatellite markers D9S171, D9S161, D17S799, and D18S474. For high molecular weight DNA, the markers D17S938 and D18S64 were also used. For paraffin-embedded samples, the markers D17S1857 and D18S1102 were used as alternatives. These markers are part of the AB1 Prism, Linkage Mapping Set, ver. 1 and 2 (Perkin Elmer). PCR products were pooled and electrophoresed on an AB1 Prism 377 instrument. Electropherograms were analysed for microsatellite alterations using GeneScan v. 3.1 and Genotyper v. 2.5 software (Perkin Elmer). Only microsatellites showing two distinct alleles in normal DNA were considered as informative. LOH was scored when there was loss of intensity of one allele in the tumour sample with respect to the matched allele from normal tissue and the relative intensity of the 2 alleles in the tumour DNA differed from the relative intensity in the non-neoplastic tissue DNA by a factor of at least two. All the analyses were verified by visual examination. Microsatellites showing differently sized alleles compared with their respective normal sample were scored as unstable. LOH on each chromosomal arm was scored in cases showing allelic loss in at least one informative marker for that arm.

Mutational analysis of K-ras, p16, and p53 and DPC4

All samples were analysed for mutations in exon 1 of the K-ras gene, exons 1 and 2 of p16, exons 5–9 of p53, and exons 8–11 of DPC4 by single-strand conformation polymorphism (SSCP) and direct sequencing of PCR amplified DNA fragments. PCR was performed in a volume of 10 μl using 40 ng DNA with 35 cycles. Primers for amplification of the p53 (Scarpa et al, 1993a), p16 (Zhang et al, 1994), K-ras (Scarpa et al, 1994b) and DPC4 genes (Hahn et al, 1998) were as described. For SSCP analysis, 0.1 μl [α-32P]-dCTP (3000 Ci mmol⁻¹) was added to the amplifications. Samples were run on 5% polyacrylamide gels containing 0.125% bis-acrylamide with and without 5% glycerol at 30 W. Bands exhibiting aberrant migration were cut from the gel, re-amplified, and sequenced on an AB1 Prism 377 instrument. The DNA from 6 xenografted control samples were a kind gift of Dr S Hahn, University of Bochum, Germany (Hahn et al, 1996).
Additional analysis for DPC4 mutations

As we found a relatively low number of DPC4 mutations using the PCR-amplified fragments described (Hahn et al, 1998), a second round of PCR-SSCP for exons 8–11 was carried out using smaller amplified fragments to verify the sensitivity of SSCP. Exons 8–11 were each divided into two fragments, each less than 180 bp. The primers used and the size of the amplified fragments were as follows. Exon 8A (167 bp): DPC4Ex8B, AAGGCTTAATATCTTCTCATGG, DPC4AS18, GAAGGTTGCAACGTAATCCAT; Exon 8B (157 bp): DPC4S18, TTTGGGTCCTCCTCCCTAT, DPC4AS8, CAAATTTTTTAAATGACTATC TGA; Exon 9A (175 bp): DPC4Ex9B, CTATACATCTGGAAGAG TAAAATT, DPC4AS19, AGTAGTAAACTGTTGACAAAAG; Exon 9B, (173 bp): DPC4S19, TTGGGTCAGGTCCTTATAGT, DPC4Ex9, TTTTGAACAAACAAAAAGCTTTAAGCT; Exon 10A (145 bp): DPC4Ex10, GAATTTTTTATGAATCTGATAG, DPC4AS10, GGAGTTTCTCTGCCAGCGG; Exon 10B (155 bp): DPC4S10, CACAAGCTGCAGCAGCTGCC, DPC4 Ex10B, ATCAACTGATATTACAGAATA; Exon 11A (179 bp): DPC4S11B, TCACCCGCCTCTCCTGAGT, DPC4AS11, AGTGAATTACGCTACCA; Exon 11B (176 bp): DPC4 S11, TTACCCCAAGACAGAGGCTCA, DPC4Ex11, TATTTTGAGTGCACCACATC.

Methylation analysis of p16

Methylation-specific PCR for the 5' CpG island of the p16 gene was carried out as described (Herman et al, 1996). DNA from the cell-line PaTul (kindly provided by Dr M von Bulow, University of Mainz, Germany) was used as a positive control and for evaluation of the sensitivity of methylation-specific PCR under the conditions used.

Immunohistochemistry for p16 and DPC4

Immunohistochemistry for p16 was performed using 3 different mouse mononclonal antibodies (p16 Ab G175–405 from BD PharMingen, San Diego, USA; Neomarker p16 Ab 8B-4 (clone 16P04) from Lab Vision Corporation, Fremont, USA; Santa Cruz p16 (F–12) from Autogen Bioclear, Calne, UK) at dilutions from 1:10 to 1:100 using different antigen retrieval and signal enhancing procedures. Immunohistochemistry for DPC4/Smad4 was performed using a mouse monoclonal antibody (Santa Cruz Smad4 B-8) from Autogen Bioclear, Calne, UK) at a dilution of 1:100 following antigen retrieval by microwaving for 3 times 10 min each in 10 mM citrate buffer, pH 6.

The immunolabelling for DPC-4 was scored as positive or negative. Tumours scored as positive showed diffuse cytoplasmic staining of the tumour epithelium and had scattered positive nuclei. Tumours scored as negative showed no detectable cytoplasmic or nuclear DPC-4 protein. Normal pancreatic structures in the same sections served as positive controls. Cases with focal loss of expression were scored as focal positive.

RESULTS

All 112 primary tumours were analysed for mutations in the DPC4 and p16 genes. Representative SSCP analyses are shown in Figures 1 and 2. The p16 gene was also examined for methylation of its 5' CpG island by methylation-specific PCR, a representative example of which is shown in Figure 3. Under the conditions used, p16 methylated sequences could be detected when they represented 2% or greater of the total input DNA (data not shown). All 112 tumours were presently or previously analysed for mutations in K-ras and p53 genes. The 53 previously reported cases included 18 PDC, 4 IPMT, 15 A VC and 16 PET (details in Materials and Methods). All tumours were also examined for allelic loss on chromosomal arms 9p, 17p and 18q at sites linked to p16, p53 and DPC4, respectively. Representative examples of this analysis are shown in Figure 4.

The status of p16 and DPC4 genes was also analysed by immunohistochemistry. However, none of the 3 anti-p16 antibodies consistently stained any structure or cell in normal pancreas with the exception of the Ab-4 from Neomarkers, which only showed positivity in variable portions of the islets of Langerhans (data not shown) confirming previous observations (Nielsen et al, 1999). Thus, the analysis of p16 status by immunohistochemistry was not feasible, as any observed negativity in neoplastic cells could not be interpreted in the absence of reliable positive controls (see also: Wilentz et al, 1998; Geradts et al, 2000). On the other hand, the anti-DPC4 antibodies efficiently immunostained all normal pancreatic acinar, ductal and islet cells, which showed strong to intermediate cytoplasmic reactivity with occasional nuclear labelling. Representative examples of immunohistochemistry with anti-DPC4 antibodies are shown in Figure 5.

Ductal carcinomas

The results of the analysis of 34 cases are summarized in Table 1. Mutations in K-ras and p53 were found in 82% and 53% of cases, respectively. Either methylation or mutation of the p16 gene was observed in 38% of cases. All mutations were somatic in nature with the exception of the p16 gene alteration in case PDC16, which was germline in origin (Moore et al, 2000b). Mutations in DPC4 were found in 9% of cases. Two recently identified polymorphisms in the DPC4 gene were also found (Table 1) (Moore et al, 2000a). To rule out the possibility that we were not able to detect sequence variants with the PCR fragments used for SSCP, each exon was subsequently analysed using two fragments less than 180 bp in length and direct DNA sequencing was performed. No additional mutations were found. As a further control, we were able to detect all DPC4 mutations by our PCR-SSCP conditions used. In 6 xenografted PDC (data not shown) kindly furnished by Dr S Hahn (Hahn et al, 1996). Allelic loss on chromosomal arms 9p, 17p, and 18q was found in 67%, 77%, and 65% of cases, respectively (Table 1). Immunohistochemical staining for DPC4 revealed that 14 cases (47%) were negative for the protein, including one case showing focal positivity. All the remaining 16 cases tested had diffusely positive labelling (Fig. 5).

Intraductal-papillary-mucinous, solid-pseudopapillary and acinar tumours

The data regarding these exocrine nonductal tumours are summarized in Table 2. In the 10 IPMT, 3 cases were found to have K-ras and p53 were found in 82% and 53% of cases, respectively. Either methylation or mutation of the p16 gene was observed in 38% of cases. All mutations were somatic in nature with the exception of the p16 gene alteration in case PDC16, which was germline in origin (Moore et al, 2000b). Mutations in DPC4 were found in 9% of cases. Two recently identified polymorphisms in the DPC4 gene were also found (Table 1) (Moore et al, 2000a). To rule out the possibility that we were not able to detect sequence variants with the PCR fragments used for SSCP, each exon was subsequently analysed using two fragments less than 180 bp in length and direct DNA sequencing was performed. No additional mutations were found. As a further control, we were able to detect all DPC4 mutations by our PCR-SSCP conditions used. In 6 xenografted PDC (data not shown) kindly furnished by Dr S Hahn (Hahn et al, 1996). Allelic loss on chromosomal arms 9p, 17p, and 18q was found in 67%, 77%, and 65% of cases, respectively (Table 1). Immunohistochemical staining for DPC4 revealed that 14 cases (47%) were negative for the protein, including one case showing focal positivity. All the remaining 16 cases tested had diffusely positive labelling (Fig. 5).
Ampulla of Vater cancers

The results of the mutational analysis in the 16 AVC are summarized in Table 3. Mutations of K-ras and p53 were found in 47% and 60% of cases, respectively. One case had a mutation of the DPC4 gene (6%), while 4 cases (25%) had inactivation of p16, all of which were due to de novo methylation (Fig. 3). Immunohistochemical staining for DPC4 revealed that 6 cases (38%) were negative, including one case showing focal positivity. The remaining 10 cases were all diffusely positive. Allelic loss on chromosomal arms 9p, 17p, and 18q was detected in 13%, 63%, and 50% of cases. Our genetic and immunohistochemical analysis showed that nine of 16 AVC had alterations in at least 2 of the 4 genes analysed, and that all cases showing either p16 or DPC4 alteration always showed alteration of either K-ras or p53.

Endocrine tumours

The data on the endocrine tumours is shown in Table 4. One nonfunctioning (NF) case had a mutation in K-ras and another NF tumour had a mutated p53 gene (Beghelli et al, 1998). One insulinoma was found to harbour a p16 mutation affecting a splice junction (Fig. 2). 4 patients (10%) had the A148T polymorphism in the p16 gene. No alterations were found in the remaining 10 cases. One case had a mutation in K-ras, sequence analysis of a band not showing altered migration (top panel) and of the band displaying aberrant migration. The sequence substitution is indicated by an arrow. The T>A substitution (g152) results in a splicing error (Fig. 2). 4 patients (10%) had the A148T polymorphism in the p16 gene. The other 6 patients were negative for K-ras mutations.

DISCUSSION

The results of the present extensive molecular analysis of different primary pancreatic tumour types may be summarized as follows:

a) a relatively high frequency of DNA sequence alterations of K-ras, p53 and p16 was detected in our panel of PDC, which is a result largely concordant with previous studies on primary or xenografted PDC; b) the detection of sequence mutations of DPC4 in PDC is at variance with a previous report on primary PDC and in agreement with the findings with xenografted PDC; c) alterations in K-ras, p53, p16 and DPC4 were found in a proportion of AVC and were virtually absent in PET, PAC, SPT or IPMT, the latter only showing relatively frequent K-ras mutations; d) the inactivation of DPC4 gene by additional mechanisms such as homozygous deletion could be addressed indirectly by using immunohistochemistry, which showed the absence of the protein in about half of PDC and AVC and its expression in PAC, SPT, IPMT or PET. The extreme rarity of p16 alterations and the lack of DPC4 inactivation in PET are at variance with previous reports.

Primary ductal cancers

As expected, in our series of 34 primary PDC we found mutations in K-ras and p53 at a frequency largely concordant with those observed in previous studies on primary cancers (Almoguera et al, 1988; Hruban et al, 1993; Lemoine et al, 1992; Pellegata et al, 1994; Redston et al, 1994; Scarpa et al, 1993a, 1994a). Alterations of the p16 gene were found in 13/34 (38%) cases, including 8 mutations (23%) and 5 cases with promoter methylation (15%). The p16 mutational frequency of 23% in our cases is...
in good agreement with the 17% mutational frequency found in an earlier study on 30 primary PDC (Huang et al, 1996) and reasonably close to the 35% found in 40 xenografts (Rozenblum et al, 1997). The present study is the first to address the frequency of p16 promoter methylation in primary PDC and the results are in excellent agreement with the 15–18% observed in xenografted PDC and cell-lines (Schutte et al, 1997; Ueki et al, 2000). Mutations in DPC4 were found in 9% of cases, at variance with the only other study using primary cancers where no mutations were found in 45 PDC (Bartsch et al, 1999a) and in agreement with the 13% mutations found in a total of 45 xenografts (Rozenblum et al, 1997; Villanueva et al, 1998).

Homozygous deletions are frequent inactivating events of p16 and DPC4 that can be easily detected in xenografted cancers, cell lines or short term cultures (Caldas et al, 1994; Hahn et al, 1996; Huang et al, 1996; Villanueva et al, 1998; Jonson et al, 1999). Microdissected primary cancers are well suited for microsatellite analysis and enabled us to demonstrate unequivocal allelic losses on 9p, 17p and 18q in PDC in a high proportion of cases. However, it is particularly problematic to assay primary PDC for homozygous deletion, as their detection would require a cancer cellularity approaching 100%. This cannot be achieved in the large majority of PDC (compare panels A and B of Figure 4) due to the high admixture with nonneoplastic cells.

Immunohistochemistry however could be used as an alternative to reveal p16 and DPC4 inactivation due to homozgyous deletion. Unfortunately, the status of p16 could not be reliably assessed by this method. In fact, we have used 3 different antibodies with different antigen-retrieval methods and enhancing procedures, all with unsatisfactory results. It is also worth mentioning that in the report from Wilentz et al, no normal pancreatic cell or structure was immunostained, only a number of cells in hyperplastic ductal epithelia were positive and the authors themselves reported difficulty in interpretation due to significant cytoplasmic background staining (Wilentz et al, 1998). Similar problems with p16 immunohistochemistry using 4 different commercial antibodies has also been recently reported (Geradts et al, 2000). However, immunohistochemical staining for DPC4 consistently found positive staining in all normal pancreatic acinar, ductal and islet cells. Of particularly interest, 47% of PDC showed negative staining for the protein. DPC4 immunohistochemistry has been recently shown to correlate with inactivation of the gene in more than 90% of cases (Wilentz et al, 2000). This would provide additional evidence that homozygous deletion is a major mechanism of DPC4 inactivation in ductal cancers.

Intraductal papillary mucinous tumours

To date, K-ras mutations have been found in IPMT at varying frequency, ranging from 30 to 70% (Sessa et al, 1994; Satoh et al, 1996; Kondo et al, 1997; Z’Graggen et al, 1997). p53 mutations are rare and associated with the invasive component of the tumour (Sessa et al, 1994). In 10 cryostat-enriched cases of IPMT, 7 of which had an invasive cancer component, we found 3 K-ras mutations and no alteration of p16, p53 or DPC4. It would seem reasonable to consider IPMT a tumour entity with molecular targets involved in its pathogenesis distinct from those of ductal carcinoma. The low frequency of LOH found on chromosomal arms 9p, 17p, and 18q further substantiates this supposition. As expected from these studies, all cases also stained positively for DPC4.

Acinar cancers

In PAC, mutations in K-ras are exceedingly rare (Hoorens et al, 1993; Terhune et al, 1994) and p53 mutations have not been found in 3 cases previously reported (Pellegata et al, 1994). Our 6 PAC showed no K-ras nor p16 mutations and frequent allelic losses on chromosomal arms 17p and 18q. However, our mutational analysis showed that the targets of these chromosomal losses are not p53 nor DPC4, the latter result being additionally substantiated by immunohistochemistry.
Solid pseudopapillary tumours

Neither ras gene family mutations nor alterations in p53 have been found in SPT (Lee et al, 1997; Bartsch et al, 1998). Our 5 cases confirmed this data. We found no alterations in either p16 or DPC4 and no allelic losses on either 9p, 17p or 18q. Immunohistochemistry showed positive staining for DPC4, as expected. Thus, the molecular events leading to this peculiar entity remain elusive.

Ampulla of Vater cancers

Our series of 16 AVC was composed of small neoplasms (<3 cm) of unequivocal ampullary origin, and cases showing microsatellite instability were excluded (Achille et al, 1997). Alterations were found in all 4 genes, with p53 mutations being the most frequent event (60%), accompanied in the majority of cases by the loss of the second allele. K-ras mutations were detected in 47% of cases. One case had a mutation in the DPC4 gene (6%) accompanied by the loss of the second allele, while 4 cases had inactivation of p16 (25%) by promoter methylation. The most frequent allelic losses were on chromosomal arm 17p (63%), which has recently been found to be an independent prognostic factor among ampullary cancers at the same stage (Scarpa et al, 2000). Allelic loss on chromosomal arm 18q was a relatively frequent event (50%), while 9p LOH was infrequent (13%). Interestingly, 38% of cases showed negative staining for DPC4. This would imply that homozygous deletion of the DPC4 gene is a frequent inactivating event in these tumours as for ductal cancers and reinforces the hypothesis that these two tumour types share common molecular pathways related to tumorigenesis and, possibly, progression of malignancy.

Table 1: Molecular alterations of K-ras, p53, p16 and DPC4 in primary pancreatic ductal cancers

| K-ras | Predicted effect | Prediceted alteration | Allelic Loss § | p53 | Predicted effect | Allelic Loss § | p16 | Predicted effect | Allelic Loss § | DPC4 | Predicted effect | Allelic Loss § | IHC** |
|-------|-----------------|----------------------|---------------|-----|-----------------|---------------|-----|-----------------|---------------|------|-----------------|---------------|-------|
| PDC3  | c35G>A          | G12D                 | frameshift    | LOH | none            | Nl            | none| none            | Nl            |      |                 |               |       |
| PDC4  | c35G>A          | G12D                 | ret           | none| ret             | none          | none| ret             | none          |      |                 |               |       |
| PDC5  | c35G>T          | c471-476del5bp       | ret           | none| none            | c629insT      | frameshift    | LOH | none            | Nl            |      |                 |               |       |
| PDC6  | c35G>T          | G12V                 | ret           | none| ret             | none          | none| ret             | none          |      |                 |               |       |
| PDC7  | c35G>T          | G12V                 | ret           | none| ret             | none          | none| ret             | none          |      |                 |               |       |
| PDC8  | c35G>T          | G12V                 | ret           | none| ret             | none          | none| ret             | none          |      |                 |               |       |
| PDC9  | c35G>T          | G12V                 | ret           | none| ret             | none          | none| ret             | none          |      |                 |               |       |
| PDC10 | c35G>T         | G12V                 | ret           | none| ret             | none          | none| ret             | none          |      |                 |               |       |
| PDC11 | c35G>A          | G12S                 | ret           | none| ret             | none          | none| ret             | none          |      |                 |               |       |
| PDC12 | c35G>T          | G12V                 | ret           | none| ret             | none          | none| ret             | none          |      |                 |               |       |
| PDC13 | c35G>T          | G12V                 | ret           | none| ret             | none          | none| ret             | none          |      |                 |               |       |
| PDC14 | c35G>T          | G12V                 | ret           | none| ret             | none          | none| ret             | none          |      |                 |               |       |
| PDC15 | c35G>A          | G12D                 | ret           | none| ret             | none          | none| ret             | none          |      |                 |               |       |
| PDC16 | c35G>T          | G12V                 | ret           | none| ret             | none          | none| ret             | none          |      |                 |               |       |
| PDC17 | c35G>T          | G12V                 | ret           | none| ret             | none          | none| ret             | none          |      |                 |               |       |
| PDC18 | c35G>T          | G12V                 | ret           | none| ret             | none          | none| ret             | none          |      |                 |               |       |
| PDC19 | c35G>T          | G12V                 | ret           | none| ret             | none          | none| ret             | none          |      |                 |               |       |
| PDC20 | c35G>A          | G12D                 | ret           | none| ret             | none          | none| ret             | none          |      |                 |               |       |
| PDC21 | c35G>A          | G12D                 | ret           | none| ret             | none          | none| ret             | none          |      |                 |               |       |
| PDC22 | c35G>T          | G12V                 | ret           | none| ret             | none          | none| ret             | none          |      |                 |               |       |
| PDC23 | c35G>T          | G12V                 | ret           | none| ret             | none          | none| ret             | none          |      |                 |               |       |
| PDC24 | c35G>T          | G12V                 | ret           | none| ret             | none          | none| ret             | none          |      |                 |               |       |
| PDC25 | c35G>T          | G12V                 | ret           | none| ret             | none          | none| ret             | none          |      |                 |               |       |
| PDC26 | c35G>T          | G12V                 | ret           | none| ret             | none          | none| ret             | none          |      |                 |               |       |
| PDC27 | c35G>A          | G12D                 | ret           | none| ret             | none          | none| ret             | none          |      |                 |               |       |
| PDC28 | c35G>A          | G12D                 | ret           | none| ret             | none          | none| ret             | none          |      |                 |               |       |
| PDC29 | c35G>A          | G12D                 | ret           | none| ret             | none          | none| ret             | none          |      |                 |               |       |
| PDC30 | c35G>A          | G12D                 | ret           | none| ret             | none          | none| ret             | none          |      |                 |               |       |
| PDC31 | c35G>A          | G12D                 | ret           | none| ret             | none          | none| ret             | none          |      |                 |               |       |
| PDC32 | c35G>A          | G12D                 | ret           | none| ret             | none          | none| ret             | none          |      |                 |               |       |
| PDC33 | c35G>T          | G12V                 | ret           | none| ret             | none          | none| ret             | none          |      |                 |               |       |
| PDC34 | c35G>T          | G12V                 | ret           | none| ret             | none          | none| ret             | none          |      |                 |               |       |
| PDC35 | c34G>C          | G12R                 | ret           | none| ret             | none          | none| ret             | none          |      |                 |               |       |
| PDC36 | c34G>C          | G12R                 | ret           | none| ret             | none          | none| ret             | none          |      |                 |               |       |
| PDC37 | c35G>A          | G12D                 | ret           | none| ret             | none          | none| ret             | none          |      |                 |               |       |
| PDC38 | c35G>A          | G12D                 | ret           | none| ret             | none          | none| ret             | none          |      |                 |               |       |
| PDC39 | c35G>T          | G12V                 | ret           | none| ret             | none          | none| ret             | none          |      |                 |               |       |

*Mutations of K-ras and p53 and allelic loss data on selected tumours are from Scarpa et al, 1993 and Achile et al, 1996. See Materials and methods for details.

**IHC immunohistochemistry: +, positive staining neoplastic cells; -, negative staining neoplastic cells; +/- focal positive staining. § LOH, loss of heterozygosity: ret, retention of alleles; Nl, noninformative.
## Table 2  Molecular alterations of K-ras, p53, p16 and DPC4 in primary pancreatic exocrine nonductal tumours*

| K-ras | p53 | p16 | DPC4 |
|-------|-----|-----|------|
| Alteration | Predicted product | Alteration | Predicted product | Allelic Loss § | Alteration | Predicted product | Allelic Loss § | IHC*** |
| IPMT1** | none found | none found | ret | none found | ret | none found | ret | + |
| IPMT2** | none found | none found | ret | none found | ret | none found | ret | + |
| IPMT3** | none found | none found | ret | none found | ret | none found | ret | + |
| IPMT4* | c35G>A | G12D | ret | none found | ret | none found | not done | |
| IPMT5*** | none found | none found | ret | none found | ret | none found | ret | + |
| IPMT6* | none found | none found | ret | none found | ret | none found | ret | + |
| IPMT7** | none found | none found | ret | none found | ret | none found | ret | + |
| IPMT8** | none found | none found | LOH | none found | ret | none found | not done | |
| IPMT9* | c35G>T | G12V | none found | LOH | none found | ret | none found | ret | + |
| IPMT10** | c35G>A | G12D | none found | LOH | none found | ret | none found | ret | + |
| IHC*** | 3/10 | 0/10 | 2/10 | 0/9 | 0/10 | 1/10 | 0/7 | 10% |
| Acinar carcinoma | | | | | | | | |
| PAC1 | none found | none found | ret | none found | ret | none found | LOH | not done |
| PAC2 | none found | none found | LOH | none found | LOH | none found | ret | + |
| PAC3 | none found | none found | ret | none found | ret | none found | ret | + |
| PAC4 | none found | none found | LOH | none found | ret | none found | ret | + |
| PAC5 | none found | not done | none found | not done | none found | not done | not done | + |
| PAC6 | none found | none found | ret | none found | ret | none found | ret | – |
| IHC*** | 0/6 | 0/6 | 2/4 | 0/6 | 1/4 | 0/6 | 2/5 | 0/5 |
| Solid-pseudopapillary tumour | | | | | | | | |
| SPT1 | none found | none found | ret | none found | ret | none found | LOH | not done |
| SPT2 | none found | none found | ret | none found | ret | none found | ret | + |
| SPT3 | none found | none found | ret | none found | ret | none found | ret | + |
| SPT4 | none found | none found | not done | not done | not done | not done | not done | + |
| SPT5 | none found | none found | ret | none found | ret | none found | ret | + |
| IHC*** | 0/5 | 0/5 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 |

*Mutations of K-ras and p53 on selected IPMT are from Sessa et al, 1994. See Materials and methods for details. **IPMT, Intraductal papillary-mucinous tumour: *, borderline; **, mucinous cancer component: *** ductal cancer component. ***IHC, immunohistochemistry; +, positive staining neoplastic cells; –, negative staining neoplastic cells; +/- focal positive staining. § LOH, loss of heterozygosity; ret, retention of alleles; Nl, noninformative.

## Table 3  Molecular alterations of K-ras, p53, p16 and DPC4 in ampulla of Vater cancers*

| K-ras | p53 | p16 | DPC4 |
|-------|-----|-----|------|
| Alteration | Predicted effect | Alteration | Predicted effect | Allelic Loss § | Alteration | Predicted effect | Allelic Loss § | IHC*** |
| AVC1 | none | c73G>A | G245D | LOH | none | ret | none | ret | LOH + |
| AVC3 | none | c659A>G | Y220C | LOH | methylated | absent | ret | none | ret | LOH – |
| AVC4 | c35G>A | G12D | none | ret | none | methylated | absent | ret | none | ret + |
| AVC5 | none | c73G>C | G245R | LOH | methylated | absent | ret | none | ret – |
| AVC6 | none | c53G>T | H179Y | LOH | none | ret | none | ret | – |
| AVC7 | none | none | ret | none | ret | none | ret | + |
| AVC8 | none | c657delC | frameshift | LOH | none | LOH | none | LOH + |
| AVC9 | c35G>C | G12A | c517G>A | V173M | ret | none | ret | none | ret + |
| AVC10 | c35G>C | G12D | c637G>T | R213X | LOH | c442G>A | polymorphism | ret | none | LOH – |
| AVC11 | c35G>A | G12D | none | LOH | methylated | absent | ret | none | LOH – |
| AVC12 | c35G>A | G12D | c524G>A | V175H | ret | none | ret | none | ret + |
| AVC13 | c35G>A | G12D | c524G>A | V175H | ret | none | ret | none | ret + |
| AVC14 | c35G>A | G12D | c524G>A | V175H | ret | none | ret | none | ret + |
| AVC15 | c35G>A | G12D | c524G>A | V175H | ret | none | ret | none | ret + |
| AVC16 | c35G>A | G12D | c524G>A | V175H | ret | none | ret | none | ret + |
| AVC17 | c35G>A | G12D | c524G>A | V175H | ret | none | ret | none | ret + |
| AVC18 | c35G>A | G12D | c524G>A | V175H | ret | none | ret | none | ret + |
| AVC19 | c35G>A | G12D | c524G>A | V175H | ret | none | ret | none | ret + |
| AVC20 | c35G>A | G12D | c524G>A | V175H | ret | none | ret | none | ret + |
| IHC*** | 7/15 | 9/15 | 10/16 | 4/16 | 2/16 | 1/16 | 8/16 | 6/16 |
| 47% | 60% | 63% | 25% | 13% | 6% | 50% | 38% |

*Mutations of K-ras and p53 and some allelic loss data on tumours AVC1-60 are from Scarpa et al, 2000. See Materials and methods for details. **IHC, immunohistochemistry; +, positive staining neoplastic cells; –, negative staining neoplastic cells; +/- focal positive staining. § LOH, loss of heterozygosity; ret, retention of alleles; Nl, noninformative.
### Table 4  Molecular alterations of K-ras, p53, p16 and DPC4 in pancreatic endocrine tumours*

| K-ras | p53 | p16 | DPC4 |
|-------|-----|-----|------|
| Alteration | Predicted | Alteration | Predicted | Allelic | Alteration | Predicted | Allelic | Alteration | Predicted | Allelic |
| Alteration product | Loss § | Alteration product | Loss § | product | Alteration product | Loss § | product | Alteration product | Loss § |
| NF1 | none | none | ret | none | ret | none | LOH + |
| NF2 | none | none | LOH | none | ret | none | LOH + |
| NF3 | none | none | ret | none | ret | none | ret + |
| NF4 | none | none | LOH | none | ret | none | ret + |
| NF5 | none | none | ret | none | ret | none | ret + |
| NF6* | none | none | LOH | none | ret | none | ret + |
| NF7* | none | none | LOH | c442G>A polymorphism | ret | none | LOH + |
| NF8* | none | none | LOH | none | ret | none | ret + |
| NF9** | none | c709delG frameshift | LOH | none | LOH | none | LOH + |
| NF10* | c35G>A G12D | none | LOH | none | LOH | none | LOH + |
| NF17* | none | none | ret | none | LOH | none | ret + |
| NF18* | none | none | ret | c442G>A polymorphism | LOH | none | ret not done |
| NF19 | none | none | LOH | none | NI | none | ret + |
| NF20* | none | none | ret | none | LOH | none | ret + |
| NF21* | none | none | LOH | none | ret | none | LOH not done |
| NF22** | none | none | ret | none | ret | none | LOH + |
| NF23 | none | none | ret | none | ret | none | LOH + |
| NF24* | none | none | ret | none | ret | none | ret + |
| NF25 | none | none | ret | none | LOH | none | LOH + |
| NF26* | none | none | LOH | none | NI | none | ret + |
| NF27 | none | none | LOH | none | LOH | none | ret + |
| NF28 | none | none | LOH | none | ret | none | ret + |
| NF29** | none | none | ret | none | LOH | none | ret + |
| NF30 | none | none | ret | none | ret | none | ret not done |
| NF31 | none | none | LOH | none | ret | none | ret + |
| NF32 | none | none | LOH | none | ret | none | ret + |
| NF33 | none | none | LOH | none | ret | none | LOH + |
| NF34 | none | none | ret | none | LOH | none | ret + |
| NF35 | none | none | ret | none | LOH | none | ret + |
| NF36 | none | none | ret | c442G>A polymorphism | ret | none | ret + |

| 1/30 | 1/30 | 15/30 | 0/30 | 11/28 | 0/30 | 9/30 | 0/27 |
| 3% | 3% | 50% | 0% | 39% | 0% | 30% | 0% |
| F1 | none | none | ret | none | NI | none | LOH + |
| F2 | none | none | ret | none | LOH | none | ret + |
| F3 | none | none | ret | none | ret | none | LOH + |
| F4 | none | none | ret | none | ret | none | ret + |
| F5 | none | none | ret | none | ret | none | ret + |
| F6 | none | none | ret | none | ret | none | LOH + |
| F11 | none | none | ret | g152T>A splicing error | ret | none | ret not done |
| F12 | none | none | ret | c442G>A polymorphism | NI | none | ret not done |
| F13 | none | none | ret | c442G>A polymorphism | ret | none | LOH + |
| F14* | none | none | ret | none | ret | none | LOH + |
| F15** | not done | not done | not done | none | not done | none | not done + |

| 0/10 | 0/10 | 0/10 | 1/11 | 1/8 | 0/11 | 4/10 | 0/9 |
| 9% | 13% | 0% | 9% | 13% | 0% | 9% | 13% |

*NF, nonfunctional; F, functional; ^ViPoma; ^^Gastrinoma. Mutations of K-ras and p53 and some allelic loss data on cases NF1–10 and F1-7 are from Beghelli et al, 1998. See materials and methods for details. **IHC, immunohistochemistry; +, positive staining neoplastic cells; –, negative staining neoplastic cells; +/- focal positive staining. Malignant case (see Methods). Malignant case with liver metastasis. § LOH, loss of heterozygosity; ret, retention of alleles; Ni, noninformative.

### Endocrine tumours

Our data confirm the extreme rarity of mutations in K-ras and p53 in these tumours (Beghelli et al, 1998; Ebert et al, 1998; Lam and L.O., 1998). The rarity of p53 mutations accompanied by LOH on chromosome 17p in 50% of nonfunctional cases, but in none of the functional PET, supports a previous suggestion indicating the presence of a tumour suppressor gene other than p53 on chromosomal arm 17p involved in nonfunctional PET tumourigenesis (Beghelli et al, 1998). The virtual absence of p16 and DPC4 alterations in our 41 cases was somewhat unexpected given the recent reports of high frequency of inactivation of these genes in PET (Muscarella et al, 1998; Bartsch et al, 1999b). In our 41 PETs, only one insulinoma showed a p16 alteration and no case showed alteration in the DPC4 gene, in spite of the finding of a moderately frequent LOH on chromosomal arms 9p and 18q, found in 39% and 30% of nonfunctional and 13% and 40% of functional tumours, respectively.

In a study of 12 PETs, the p16 gene has been reported to be altered in 92% of cases (7/8 gastrinomas and 4/4 nonfunctional) by either methylation (58%) or homozygous deletion (42%)

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*PS Moore et al, British Journal of Cancer (2001) 84(2), 253–262 © 2001 Cancer Research Campaign*
(Muscarella et al, 1998). In our series, only one insulinoma had a mutation in p16 and none of the 41 PETs showed p16 methylation. The sensitivity of our methylation-specific PCR rules out the possibilities that tumour heterogeneity or neoplastic cellularity might have biased this result. It is possible that p16 inactivation by promoter methylation might be restricted to functional gastrinomas, as all the reported PETs showing p16 methylation were of this subtype (Muscarella et al, 1998). Moreover, neoplastic cellularity is not as problematic in PET with respect to PDC and virtually all cases can be enriched to nearly 100%. We observed no homozygous deletions of the p16 gene under identical, standard amplification conditions previously used (Muscarella et al, 1998). Although it cannot be excluded that a small proportion of PETs have homozygous deletion of p16, it would appear to be a rare event. Finally, although the A148T polymorphism in the p16 gene was found in 10% of patients, which is well above the expected frequency (1.8%) (Aitken et al, 1999), this sequence variant has been reported to be functionally silent (Reymond and Brent 1995) and was not of statistically significant risk in families with melanoma (Aitken et al, 1999).

A recent study reported DPC4 alterations in 5 of 9 malignant nonfunctional PET and in none of 16 functional PET, where it was suggested that alteration of this gene was correlated with malignancy (Bartsch et al, 1999b). Only one of these 5 alterations resulted from homozygous deletion, with the remaining 4 being mutations. We observed neither mutations nor homozygous deletions in the DPC4 gene in our series of 11 functional and 30 nonfunctional enriched tumours. The latter included 18 malignant cases, 10 of which had liver metastases. Thus, our data suggest that the DPC4 is not likely to play a central role in neuroendocrine tumourigenesis and the previously reported data may be biased by serendipity due to the small number of cases studied (Bartsch et al, 1999b). The conclusions reached from our molecular analysis of the DPC4 gene in PET is further strengthened by results from immunohistochemistry in which all cases tested stained positively. Thus, it would appear unlikely that homozygous deletion of the DPC4 gene is a frequent event in PET.

CONCLUSIONS

By the molecular analysis of 112 pancreatic tumours of different types, it can be inferred that only carcinomas arising from the epithelium of pancreatic ducts and their terminal excretory structure (ampulla of Vater) have common molecular features. The genetic pathways implicated in ductal cancer are not involved in the pathogenesis of exocrine nonductal or endocrine tumours. These neoplasms must therefore have distinct molecular pathways involved in tumourigenesis. This likelihood seems highly plausible when considered together with the fact that each tumour type has distinct pathological and clinical features, including dramatic differences in patient survival.

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