

***INK4/ARF* germline alterations in pancreatic cancer patients**

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Background: Roughly 40% of germinal mutations in melanoma families (MF) affect p16^{INK4a} and p14^{ARF}. We investigated the association between *INK4/ARF* alterations and the occurrence of pancreatic cancer in MF and in sporadic pancreatic cancer (SPC) patients.

Patients and methods: Forty-nine MF, 66 SPC cases and 54 controls were enrolled. The *INK4/ARF* locus was screened.

Results: As compared with the general population, the risk of pancreatic cancer (PC) was increased 9.4-fold [95% confidence interval (CI) 2.7–33.4] and 2.2-fold (95% CI 0.8–5.7) in G101W-positive and -negative MF, respectively, while mean ages at onset were 61 and 77 years, respectively. A 1.7 (95% CI 1.06–2.79) increased risk of cancer at any site was observed among first-degree relatives of SPC cases as compared with controls. The G101W founder mutation was detected in 4% of SPC cases but the rate increased to 13% when tumor clustering in either branch of families was taken into account. One G101W-positive PC patient with a melanoma in a first-degree relative harbored a germline deletion of the second allele, including exon 1B.

Conclusions: The presence of a deletion including exon 1B in two PC patients points to the involvement of p14^{ARF} in the development of PC and may suggest that the increased risk of PC in MF is caused by impairment of both loci.

Key words: *CDKN2A*, familial melanoma, G101W mutation, increased risk, p14^{ARF}, pancreatic cancer

Introduction

Pancreatic cancer is the fifth leading cause of cancer death in Italy where it accounts for over 7000 deaths per year [1]. Due to the aggressive nature of the disease and the difficulties in diagnosis, treatments are chiefly palliative and 5-year survival does not exceed 5%.

Pancreatic cancer has been reported to be associated with various environmental and lifestyle risk factors, occupational exposures and medical conditions; however, the only risk factors consistently reported are age and smoking status, and the etiology of the disease remains largely unknown [2].

A positive family history of pancreatic cancer [3–5] and a history of other cancers among first-degree relatives have been established as important risk factors for the disease [6, 7]. Moreover, the presence of multiple primary tumors in pancreatic cancer patients [8] or early age at onset [9] may suggest a genetic predisposition to the development of malignancies. Several genetic syndromes, such as ataxia telangiectasia, Peutz–Jegher’s syndrome, hereditary nonpolyposis colorectal cancer and familial atypical mole-malignant melanoma syndrome (FAMMM), are also associated with a significantly increased risk of developing pancreatic

cancer [10]. In addition, an excess of pancreatic cancer has been found to occur in breast–ovarian cancer families carrying *BRCA1* and *BRCA2* mutations [11].

Among the susceptibility genes involved in the syndromes mentioned above, *CDKN2A/p16^{INK4a}* seems to play an important role. p16^{INK4a} is a negative regulator of cell cycle progression at the G₁–S restriction point and is frequently altered in sporadic pancreatic adenocarcinomas. Virtually all pancreatic carcinomas show inactivation of the *CDKN2A* gene [12, 13]. An increased risk of developing pancreatic adenocarcinoma was found in melanoma and FAMMM kindreds carrying *CDKN2A* mutations; no excess of pancreatic adenocarcinoma occurred in kindreds with wild-type *CDKN2A* [14–18].

We previously described a small number of familial melanoma kindreds from a very small area in Liguria, a northwestern region of Italy [19, 20], where the p16^{INK4a} G101W germline founder mutation is prevalent [21]. Seven of those kindreds were found to carry the G101W mutation and displayed an increased risk of pancreatic cancer. An increased risk of pancreatic cancer in melanoma families has been reported in other founder populations: Borg et al. [22] showed that the 113insArg founder mutation was associated with an increased risk of multiple melanomas, breast cancer and pancreatic cancer; Vasen et al. [23] reported that putative mutation carriers belonging to Dutch FAMMM kindreds had a 17% cumulative risk of developing pancreatic cancer by 75 years of age.

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The majority of mutations which have been associated with an increased risk of pancreatic cancer are located on exon 2 [14–20] and are thus predicted to affect the amino acid sequence of p14^{ARF} (due to alternative splicing of exon 1B with *CDKN2A* exon 2) [24]. Additionally, although few families have been reported to harbor mutations in p14^{ARF} which predispose to melanoma [25, 26], a germline deletion affecting *p14* but not *CDKN2A* in a melanoma-neural system tumor syndrome family has recently been identified [27]. Therefore, the involvement of the *INK4/ARF* locus in pancreatic cancer development deserves further study. To this purpose, and more specifically to evaluate the role of the G101W founder mutation in the development of pancreatic cancer among melanoma-prone kindreds, we extended our previous analysis to a greater number of families and to a pool of unselected pancreatic cancer patients.

Patients and methods

Incidence of pancreatic cancer in melanoma-prone kindreds

Selection criteria. From January 1995 to December 2000, 83 probands were consecutively referred to the Regional Medical Genetics Service of the University of Genoa and their familial pedigree was built. Forty-nine probands were selected for displaying two or more melanomas per family and for their Ligurian origin; of these, 42 have been previously described [28]. The remaining 34 probands were not enrolled either because they did not meet selection criteria (six were not of Ligurian origin, seven lacked confirmation for the second melanoma), or, following the educational session, they decided against DNA testing (10), or they carried a *CDKN2A* mutation other than the G101W founder mutation (one).

Features of melanoma families. The 49 melanoma families from Liguria were comprised of 967 relatives, including 118 melanoma cases. Twelve of 118 melanoma cases were excluded because information on the age of diagnosis of melanoma was not recorded ($n = 4$) or diagnosis was earlier than 1958 ($n = 8$) when no written documentation was available prior to that date. Thus, 106 familial melanoma patients diagnosed from January 1959 to December 2000 were studied from a total of 955 relatives, 366 of which belonged to the unaffected branches and were reclassified as controls (at date of analysis, 207 alive and 159 deceased); of the 589 members of the affected branches, 409 were alive and 180 deceased.

The 18 G101W families and 31 non-mutated families comprised 219 and 370 members, respectively. A total of 132 members were screened for the mutation (52 from the G101W families and 80 from non-mutated families). Thirty-four members of the 18 G101W-positive kindreds were found to harbor the mutation. The mean ages at diagnosis of melanoma in the G101W families and mutation-negative kindreds were 46.5 (median, 42; range, 20–92) and 49.4 years (median 49, range 16–85), respectively.

Verification of diagnosis

Information on melanoma patients was collected from histopathological and clinical records from different hospitals in Liguria. Once an affected family member was identified, access to the medical file and/or autopsy report was requested. If medical files and/or autopsy reports were not available, information on malignancy was obtained from the Ligurian Cancer Registry [29], which holds incidence figures from 1986 to 1996. Information on family history of pancreatic cancer was reported by patients during genetic counseling. Four out of 12 of those reports were confirmed. For two cases diagnosed before 1986 or after 1996 (two of four patients) confirmation was provided by declarations of a general physician (one patient) or an oncologist (one patient). The other two were confirmed by the local cancer registry. The remaining

eight cases were not confirmed either because they were diagnosed before 1986 (five patients), or because the patients were diagnosed in another region and no documentation was available (three patients).

Hereditary susceptibility to cancer in unselected pancreatic cancer patients from Liguria

The study was designed as a hospital-based case–control study at the National Cancer Institute (NCI) in Genoa, the largest town in Liguria, accounting for roughly 50% of the region's total population.

Cases. Eligible patients were those with a diagnosis of pancreatic cancer ≥ 18 years of age who were referred to the Gastrointestinal Unit of the National Institute for Cancer Research (NCI) of Genoa for endoscopic retrograde cholangio-pancreatography (ERCP). In all, 66 patients (36, male; 30, female) were interviewed. A histological diagnosis of exocrine pancreatic cancer was available for 23 patients (34.8%) and a cytological diagnosis was obtained for six patients (9.1%). In the remaining cases, the diagnosis was made on the basis of a diagnostic laparotomy [18 cases (27.2%)] or imaging [19 cases (28.8%)].

Two patients (one male, one female) were excluded from the analysis, as the diagnosis of pancreatic cancer was not confirmed, one male was excluded because the pathological examination showed an endocrine carcinoma and two cases (both males) were excluded because they were unable to report their family history of cancer, leaving 61 cases available for analysis.

Controls. Controls were identified at the San Martino General Hospital, Genoa, which is located close to the NCI. Subjects were eligible as controls if they had been admitted for diseases other than cancer and unrelated to digestive tract disease or to known risk factors for pancreatic cancer. Controls were matched to cases by gender, age (± 5 years) and region of residence. In all, 54 eligible controls were identified and interviewed. The main diagnostic categories for hospital admission were acute surgical ($n = 19$) and musculoskeletal diseases ($n = 35$).

A trained interviewer administered a standardized questionnaire to cases and controls. They were asked about their own disease, cancer occurrence in the family (type and site of primary tumor), age at diagnosis among first-, second- and third-degree blood-relatives. Cancer site among relatives was recorded as reported by cases and controls and no attempt was made to verify the diagnoses. An in-person interview was conducted with 48 of 61 (78.7%) cases and with 52 of 54 (96.3%) controls, for the others a family member was interviewed.

Cancer clustering in the family was hypothesized when the index case (or control) reported a history of invasive cancer in at least two blood relatives (first and/or second degree) belonging to the same branch of the family.

Cases and controls received detailed information about the purpose of the study. Written informed consent concerning participation in the study, potential risks and benefits, use of hospital information and DNA analyses was obtained. All subjects who agreed to participate in the study were asked to provide a 20 ml sample of peripheral blood to perform molecular analyses. Cases and controls were interviewed while still at the hospital. The study was approved by the Ethics Committee of the NCI of Genoa.

Molecular analysis

***INK4/ARF* mutation analysis in melanoma families and pancreatic cancer patients.** DNA was extracted from peripheral blood leukocytes using standard procedures. Specific PCR products of each of the four exons at the locus and at the intron–exon junctions were screened for mutations by single strand conformational polymorphism (SSCP) analysis, followed by direct sequencing of the variants. Primer sequences and screening conditions for exons 1 α , 2 and 3 of *CDKN2A* have been previously described [28]. Thirty-one probands from *CDKN2A*-negative families were screened for alternative exon 1 β (encoding p14^{ARF}) mutations and for the recently described IVS2-105A/G deep intronic

mutation [30] by PCR and SSCP. Primers used for exon 1 β amplification (447 bp) were p14F, 5'-TCCCAGTCTGCAGTTAAGGGG-3' and p14R, 5'-GTCTAAGTCGTTGTAACCC-3; the deep intronic variant was investigated using primers 105F, 5'-GGGTACATGCACGTGAAGC-3' and 105R, 5'-GTTTCTCAGAGCCTCTCTGGT (designed to amplify a 182 bp fragment). Positive controls (kind gift of Dr S. Puig, Barcelona, Spain) for exon 1 β 60ins16 mutation and for the IVS2-105A/G variant (kind gift of Dr M. Harland, Leeds, UK) were included for each experiment. Briefly, 60 ng DNA were amplified in a final volume of 15 μ l containing 5 pmol primers, 200 μ M dNTPs and 1.5 mM MgCl₂, 0.5 U TaqGold (Applied Biosystem, Foster City, CA) in a PE2400 mastercycler with an amplification profile of 95°C for 12 min, followed by 35 cycles at 95°C for 50 s, 60°C for 30 s, 72°C for 1 min and a final extension at 72°C for 7 min.

DNA from 48 pancreatic cancer patients was screened for both *CDKN2A* (exons 1 and 2) and exon 1B mutations as described above. Thirty-five controls were also screened for *CDKN2A* mutations.

Microsatellite analysis at the *INK4/ARF* locus. Microsatellite analysis at the *INK4/ARF* locus was performed using markers (IFNA, D9S736, D9S1749, D9S974, D9S942, D9S1748, D9S1604, D9S171, all primer oligonucleotide sequences are available at <http://www.gdb.org>). For each primer pair, one oligonucleotide was labeled with a 5' fluorescent amidite (6' FAM or HEX). Amplification was carried out using TaqGold (Applied Biosystems) and PCR products were visualized using an ABI 310 automated DNA sequencer (Applied Biosystems).

Statistical analysis

Melanoma families. The incidence of pancreatic cancers was compared with the expected incidence in the general population and among spouses of those affected (control group). The number of person-years of observation was calculated for each subject from birth until death, diagnosis of cancer, or the last known time at which the subject was alive and cancer-free. Person-years at risk were stratified according to gender and age (5-year intervals) and *p16* status (mutated or wild type). The number of expected cases, by age (5-year intervals) and gender, was calculated from Ligurian Cancer Registry data (1993–1996) [29]. The expected number of pancreatic cancer cases was calculated in each stratum by multiplying the stratum-specific incidence rate of pancreatic cancer in the Ligurian Cancer Registry by the number of person-years of observation in the study population (mutation carriers, wild type, controls). We calculated the crude incidence ratio (ratio of observed to expected number of cases); the expected number of cases was summarized to yield the total number of cases expected on the basis of the Cancer Registry. The 95% confidence intervals (CI) were calculated assuming a Poisson distribution for the observed number of melanoma or pancreatic cancer cases in the study population.

The chi square test was used to test for differences among proportions and comparison of age between groups was performed using the Mann–Whitney test.

The age-specific risk of developing pancreatic cancer was calculated according to the Kaplan–Meier method for survival analysis [31]. The end point was the diagnosis of pancreatic cancer, death or end of follow-up (December 2000) or the last date when the patient was known to be alive.

Case–control study. The relative risk of cancer among relatives of cases of pancreatic cancer and of hospital controls was estimated using the Cox proportional hazard method [32]. When appropriate, gender was introduced in the model as a covariate.

For all statistical tests a significance level of 0.05 was used, and all reported *P* values are two-sided. All analyses were performed using the statistical package SPSS for Windows, release 5 (SPSS, Inc., Chicago, IL).

Table 1. Risk of pancreatic adenocarcinoma among Ligurian melanoma families

Families	Pancreatic cancer		RR	95% CI	<i>P</i> ^a
	Observed	Expected			
All (<i>n</i> = 49)	10	2.5	4.0	2.2–7.5	<0.000
Males	5	1.3	3.9	1.6–9.4	0.001
Females	5	1.2	4.1	1.2–14.5	0.0005
Wild type (<i>n</i> = 31)	4	1.8	2.2	0.8–5.7	0.11
Males	2	0.9	2.1	0.6–6.6	0.27
Females	2	0.9	2.2	0.6–7.8	0.25
Mutation (<i>n</i> = 18)	6	0.6	9.4	2.7–33.4	0.000
Males	3	0.3	9.0	2.6–32.2	0.000
Females	3	0.3	9.9	2.8–34.9	0.000

^aAll *P* values are two-sided.

RR, relative risk; CI, confidence intervals.

Results

Risk of pancreatic adenocarcinoma among Ligurian melanoma families

Pancreatic cancer was recorded in one of 366 (0.3%) spouse controls, in four of 370 (1.1%) subjects belonging to the 31 wild-type families and in six of 219 subjects belonging to the 18 mutated families (2.7%). No case reported diagnosis of both melanoma and pancreatic cancer. In families 12 and 17 (both with G101W mutation), two cases of pancreatic cancer among blood relatives were reported.

In the three groups, other cancers were reported in 38 (10.4%), 39 (10.5%) and 19 subjects (8.7%), respectively. After melanoma, pancreatic adenocarcinoma was the second most frequent cancer in G101W-positive families.

The risk of developing pancreatic cancer among our melanoma kindreds is shown in Table 1. A statistically significant 4-fold increased risk of pancreatic cancer was observed among subjects belonging to the melanoma families (95% CI 2.2–7.5). However, when the mutational status of the family was considered, the risk of pancreatic cancer in the wild-type families fell to 2.2 (95% CI 0.8–5.7), which was not statistically significant, while among the mutated families the risk rose to 9.4 (95% CI 2.7–33.4). The Poisson regression analysis provided a 2.1-fold increased risk of pancreatic cancer (95% CI 0.6–3.2) for subjects belonging to a melanoma family which was not statistically significant. The observed risk associated with the presence of the G101W mutation in the family was 4.4 (95% CI 1.2–15.5).

The 0–74 years cumulative risk of pancreatic cancer in G101W families, in wild-type families and the general Ligurian population is reported in Figure 1: the cumulative risk was 6.7% (95% CI 0.9% to 12.5%) in the mutated families and 1.7% (95% CI 0.0% to 4.0%) in the wild-type families. The risk in the general population was 1.4%.

Subjects with pancreatic cancer belonging to mutation positive families (mean age at diagnosis of pancreatic cancer, 61 years;

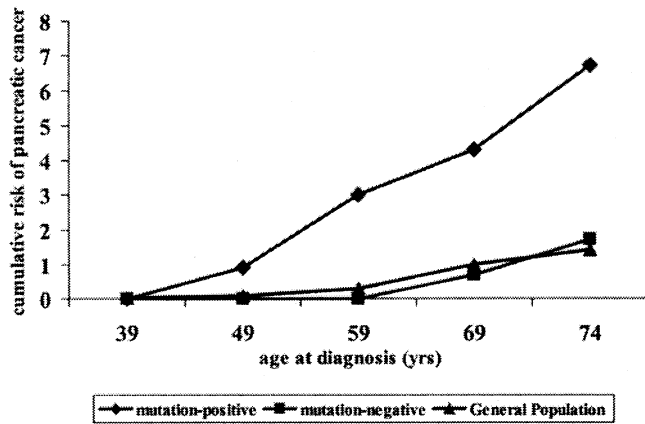


Figure 1. Cumulative risk of pancreatic cancer according to *p16* status in the family and the general population (Ligurian Cancer Registry 1996).

Table 2. Features of relatives (first, second and third degree) of case and control probands

	Relatives of cases		Relatives of controls	
	No.	Mean age (range)	No.	Mean age (range)
First degree	365	56.9 (2–96)	262	60.9 (1–96)
Parents	120	71.0 (29–96)	105	75.1 (23–96)
Siblings	154	58.8 (2–91)	103	59.9 (1–85)
Offspring	91	35.0 (8–60)	54	35.1 (10–62)
Second degree	373	55.2 (1–98)	354	57.8 (1–96)
Third degree	87	43.2 (1–80)	80	41.7 (1–80)
Total No. of relatives	825	54.7 (1–98)	696	57.1 (1–96)

range 49–78) were younger than those belonging to the wild-type families (mean age at diagnosis, 77 years; range 65–88) ($Z = 1.919$; $P = 0.055$).

Hereditary susceptibility to cancer in unselected pancreatic cancer patients

A total of 61 pancreatic cancer patients (case probands) and 54 controls (control probands) were available for analysis. Cases comprised 36 males and 25 females; the mean age at diagnosis was 64.6 years [standard deviation (SD) 10.9; range 41–83]; 67.2% were born in northern Italy. Among case probands, two patients (case nos 101 and 111) reported that they had had a histologically confirmed cutaneous malignant melanoma removed 4 and 10 years before the diagnosis of pancreatic cancer, respectively. Controls comprised 32 males and 22 females; the mean age at the time of interview was 64.4 years (SD 10.4; range 42–79); 85.2% were born in northern Italy. In all, cases and controls reported 1521 relatives, whose features are summarized in Table 2.

Case probands reported a cancer in 52 of 365 (14.2%) first-degree relatives, 49 of 373 (13.1%) second-degree relatives and

five of 87 (5.7%) third-degree relatives; the same figures reported by control probands were 26 of 262 (9.9%), 41 of 354 (11.6%) and 10 of 80 (12.5%), respectively. Among first-degree relatives, the cumulative incidence of cancer (all sites) to age 84 years was 35% for cases and 18.8% for controls ($\chi^2_1 = 5.41$, $P = 0.02$). Among second-degree relatives, the cumulative incidence of cancer was similar in cases and controls (23.9% versus 21.0%; $P = 0.85$).

The estimated RR of cancer at any site was 1.72 (95% CI 1.06–2.79; $P = 0.028$) for first-degree relatives and 1.06 (95% CI 0.67–1.67; $P = 0.81$) for second-degree relatives of cases as compared with those of controls. Concerning the most common cancer sites, first-degree relatives of cases showed a non statistically significant 2-fold increased risk of cancer of the large bowel (10 cases and four controls, RR = 2.3; 95% CI 0.7–7.4), lung (five cases and two controls, RR = 2.3; 95% CI 0.4–11.9), uterus (unspecified) (five cases and two controls, RR = 2.1; 95% CI 0.4–11.0) and aerodigestive tract (four cases and two controls, RR = 2.3; 95% CI 0.4–11.7) as compared with relatives of controls. As for rare cancers, two cases of pancreatic cancer, one case of melanoma and one of rhabdomyosarcoma were reported among first-degree relatives of cases. None of these three tumor types were recorded among first-degree relatives of controls.

We further evaluated the individual pedigree of cases and controls in order to identify cancer clustering. Separately, we analyzed the paternal and maternal lineage in the attempt to understand whether the clusters were consistent with a genetic predisposition to the development of tumors. A clustering was identified in 19 branches out of 61 case families (31.1%), and in five of 54 (9.3%) control families ($P = 0.004$).

In nine of 19 branches, we observed additional primary tumors which are not inconsistent with a partial spectrum of family cancer syndromes (melanoma-pancreas, HNPCC, breast-ovarian), or pancreatic cancer in patients with a previous diagnosis of cutaneous malignant melanoma (in two patients) or familiarity for pancreatic cancer (one patient with a first-degree relative affected by pancreatic cancer) (Table 3) (Figure 2). Pancreatic cancer was diagnosed at a younger age among patients belonging to branches with clustering (mean age at diagnosis, 60.4 years; range 41–82 years) than among those belonging to the branches with no clustering (mean age at diagnosis, 66.6 years; range 47–83) ($Z = 1.987$; $P = 0.047$).

Molecular analysis of the *INK4/ARF* locus in melanoma families and in sporadic pancreatic cancer patients

In order to verify the involvement of the *INK4/ARF* locus in melanoma development among the 31 *p16*-negative families, the melanoma probands were screened for mutations in exon 1B and in an intron 2 fragment recently described to harbor a IVS-105 A/G mutation in a subset of *p16*-negative melanoma families, and no mutation was detected.

Out of 48 pancreatic cancer patients screened for *CDKN2A* and exon 1B mutations, two were found to harbor the *CDKN2A* G101W mutation. No mutation was found in the 35 controls tested. The two pancreatic cancer patients carrying the *p16* mutation showed an association of pancreatic cancer with other tumors in first and second-degree relatives (prostate, colon, larynx and melanoma) (Figure 2B). A cumulative 4% mutation frequency

Table 3. Cancer clustering and *INK4/ARF* mutation status in families of pancreatic cancer cases and controls

Cases	Gender	Age at diagnosis	Cancer site and age at diagnosis/death		<i>INK4/ARF</i> mutation status
			First degree	Second degree	
101	M	PC 63, Mm 59	Rhabdomyosarcoma 84 ^a		Wild-type
105	F	PC 57	Colon 67	Colon 75 ^a , liver 54	–
110	F	PC 54		Bilateral breast 70 ^a , breast 60	Wild-type
111	M	PC 63, Mm 53	Colon 84 ^a		Wild-type
113	M	PC 63	Ovary 53 ^a , ovary 53 ^a		Wild-type
115	M	PC 62	Stomach 68 ^a	Stomach 75 ^a	Wild-type
120	F	PC 73		Colon 60, colon 60, CSU 60,	G101W/R115L
124	F	PC 56	PC 72, kidney 54	Bladder 50 ^a , lung 50 ^a , CSU 60 ^a , liver 63 ^a	Wild-type
126	M	PC 54	Colon 48, larynx 74 ^a		Wild-type
133	F	PC 48	Mm 48	Colon 66	G101W/R115L
136	F	PC 83	Colon 72, uterus 64, lung 77	Breast 42	Wild-type
138	F	PC 76	Breast 71, esophagus 60, lung 60		Wild-type
145	M	PC 41	Bladder 53, uterus 33, colon adenomas 39		–
150	M	PC 65	Liver 82	CNS 65 ^a , liver 50	Wild-type
154	M	PC 52		Breast 40, colon 60 ^a , lung 70 ^a	–
156	M	PC 67	Colon 83, myeloma 50, lymphoma 52		Wild-type
157	F	PC 73	Prostate 53	Colon 60, colon 60, larynx 58	Wild-type
158	M	PC 41	Leukemia 55 ^a , colon 36		Wild-type
161	F	PC 58	Breast 56	Breast 64 ^a , prostate 76 ^a , bladder 79, larynx 80	–
Controls					
228	M	Cont 47	CNS 60	Lung 59 ^a , CNS 75	Wild-type
232	F	Cont 56	Prostate 67, breast 28	Breast 85	Wild-type
248	M	Cont 73	Colon 65, breast 59	Colon 70 ^a	–
256	M	Cont 69		Colon 48 ^a , larynx 66	–
257	F	Cont 72	Colon 59 ^a , ovary 33		–

^aAge at death.

Cont, control; CSU, cancer site unknown; CNS, central nervous system; Mm, malignant melanoma; PC, pancreatic cancer.

was found among all pancreatic cancer cases analyzed, but the frequency increased to 13% (2 of 15 cluster families tested) when we observed pancreatic cancer patients from cluster families alone.

Analysis of markers flanking the *CDKN2A* locus showed that genotypes of the two pancreatic cancer patients carrying the G101W mutation were consistent with the previously reported G101W core haplotype [21], although the phase could not be determined.

One of the two patients harboring the mutation revealed a region of hemizyosity involving four markers (D9S942 to D9S171), thus suggesting a germline deletion of the wild-type allele containing exon 1B in this patient. The two patients affected by melanoma and pancreatic cancer, and found to be negative at *CDKN2A*, exon 1B and deep intronic variant screening, were also typed for microsatellite analysis. One of them carried a deletion involving markers D9S942 to D9S1604 (Table 4).

Discussion

Genetic screening of founder populations allows specifically targeted testing and should improve test yields as well as the precision of cancer risk estimates among mutation carriers. Moreover, these populations may be a powerful resource to localize other low penetrance susceptibility loci because of reduced mutational heterogeneity.

The Ligurian melanoma families described here showed a 9-fold increase in the risk of developing pancreatic cancer as compared with the general Ligurian population. These results are comparable with those reported by Vasen et al. [23]: the mean age at diagnosis of pancreatic cancer was 57 years in our study and 58 years in the Leiden study, and the cumulative risk of pancreatic cancer among non-mutated families did not differ from that in the general population. The cumulative risk among the Dutch families was higher than that observed here (17% versus 6.7%). This may

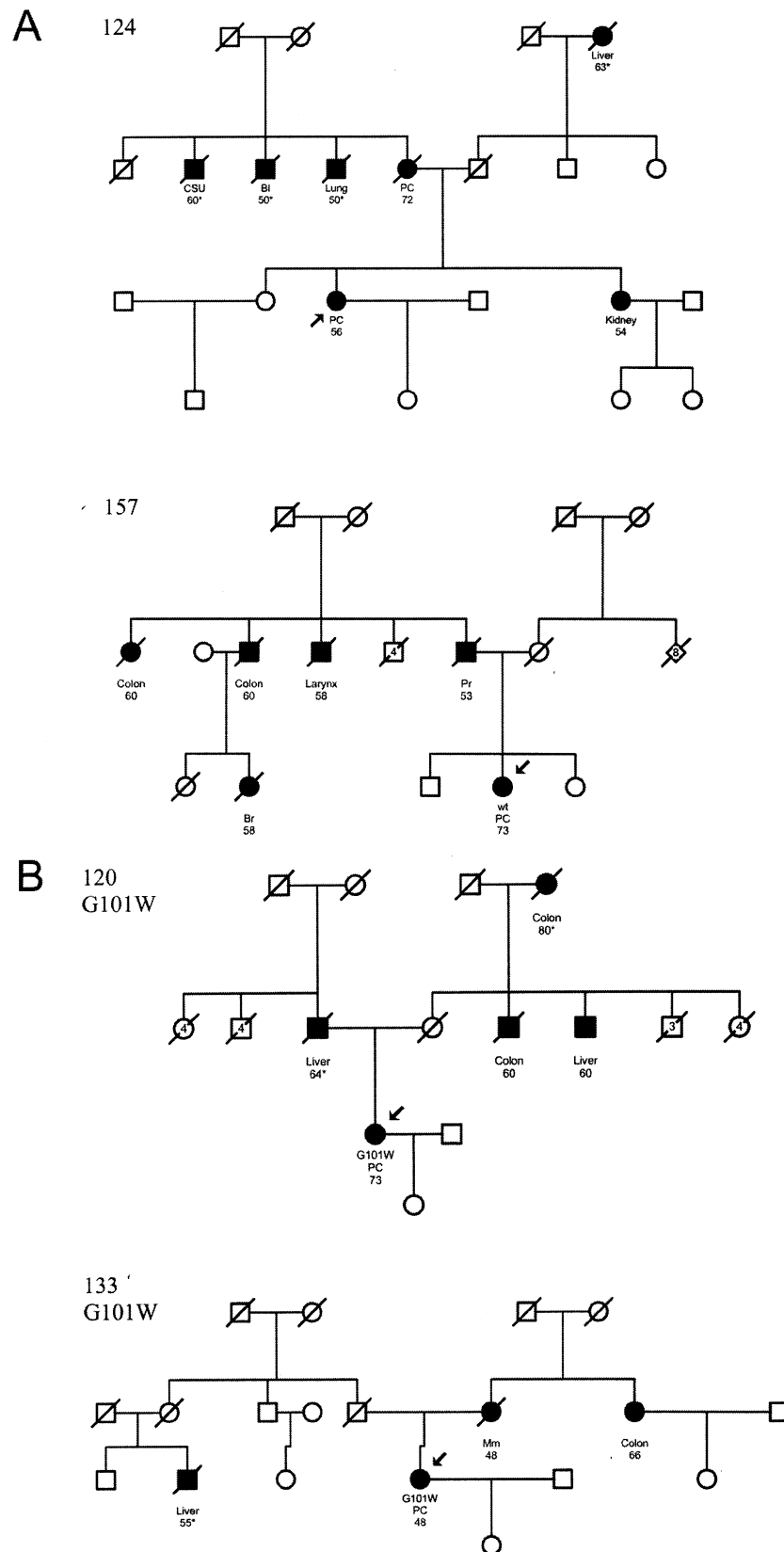


Figure 2. Pedigrees showing a partial spectrum of family cancer syndrome. Family and mutation probands are indicated by arrows. Black symbols indicate malignant disease. Age at diagnosis or age at death (*) and mutational status are indicated under each symbol. Family numbers are indicated as reported in Table 3. Bl, bladder cancer; Br, breast cancer; colon, colon cancer; CSU, cancer site unknown; kidney, kidney cancer; larynx, larynx cancer; liver, liver cancer; Mm, malignant melanoma; PC, pancreatic cancer; Pr, prostate cancer. (A) Examples of pedigrees of *p16/p14*-negative cases (wt). (B) Pedigrees of *p16* mutation-positive (G101W) cases.

Table 4. Haplotype analysis at *INK4/ARF* locus (9p21)

Markers	G101W haplotype	Case 120		Case 133		Case 111		Case 101	
IFNA	<i>149</i>	145	145	153	149	151	153	145	145
736	<i>174</i>	172	176	174	174	–	–	172	172
1749	<i>133</i>	137	139	135	145	109	125	141	143
Ex3,2,1 α	–	WT	<i>G101W</i>	WT	<i>G101W</i>	WT	WT	WT	WT
974	<i>211</i>	205	<i>211</i>	209	<i>211</i>	207	209	209	213
942	<i>114</i>	118	<i>114</i>	114	114	114	114	114	116
1748	<i>110</i>	120	<i>110</i>	110	110	120	120	112	122
ex 1 β	–	WT	<i>WT</i>	WT	WT	WT	WT	WT	WT
1604	<i>190</i>	192	<i>190</i>	190	190	190	190	190	192
171	<i>169</i>	175	<i>169</i>	161	161	161	169	169	179

Mutation carriers (cases 120 and 133) and cases with multiple primaries (pancreatic cancer and melanoma) (cases 111 and 101) were haplotyped. Italics show G101W haplotyped as described in [21] and haplotype sharing of the two G101W-positive cases. Allele numbers were assigned on the basis of actual length and 2-base-pair repeat spacings. Bold characters show regions of deletion, including exon 1 β but not *CDKN2A*.

WT, wild type.

be due to the impact of gene–environment or gene–gene interactions on mutations which in both cases are predicted to affect the role of p14^{ARF}.

As it is almost impossible to test relatives affected by pancreatic cancer in a retrospective study—due to the very poor prognosis of these patients—our analysis may suffer from a lack of genotypic information on pancreatic cancer cases. Hence, we chose to investigate family history and *INK4/ARF* mutations in unselected pancreatic cancer cases from the same geographic area.

All pancreatic cancer cases included in the case–control study were selected and reviewed by the same gastroenterologist (V.P.) who carried out the ERCP analysis. A histological or cytological diagnosis of pancreatic cancer was obtained for only 45.9% (28 of 61) of the cases enrolled in the study. So, in spite of a careful review of the clinical records, we cannot exclude misclassification of some cases. In addition, 13 patients underwent palliative surgery because of inoperable disease, and in the surgical report, a pancreatic mass involving the contiguous organs was recorded. The diagnosis was based on imaging alone in 19/61 patients (31.1%). Nonetheless, the rate of confirmation of pancreatic cancer in epidemiological studies rarely exceeds 70% [6, 33], and in a subset of an international case–control study of the International Agency for Research on Cancer (IARC) [34], the rate was as low as 43%.

Large case–control studies carried out in Italy estimated that roughly 3% of newly diagnosed pancreatic cancers are consistent with the existence of a genetic component in the familial aggregation of pancreatic cancer [35]. In our study population, the observed frequency of first-degree family history of pancreatic cancer among cases was 3.3%, which was slightly lower than that reported by other epidemiological studies [3, 7]. None of the controls reported family history of pancreatic cancer; this may be due to the low expected frequency (0.6–1.5%) of pancreatic cancer among relatives of healthy subjects [3, 7]. It should be noted that information on the family history of cancer was collected during

the interview and no attempt was made to verify the reported information. Until two decades ago, the diagnosis of pancreatic cancer was difficult because of a lack of technical devices, in addition to the massive invasion of the contiguous organs at diagnosis very frequently precluding histological diagnosis. Therefore, we cannot exclude the fact that relatives with pancreatic cancer may have been reported to have died of some other abdominal cancer (most frequently, liver cancer). However, we can rule out the recall bias among cases, as most were interviewed shortly after their ERCP examination when the diagnosis of pancreatic cancer had not yet been confirmed. In our case–control study, we observed that first-degree relatives of patients with pancreatic cancer had a statistically significant 1.7-fold increased risk of developing cancer at any site as compared with controls. The cumulative incidence of cancer (all sites) among first-degree relatives of patients with pancreatic cancer was 35% and among relatives of controls it was 18.8%. When we considered the most frequent sites of neoplastic disease, we found that first-degree relatives of patients with pancreatic cancer had a 2-fold increased risk of developing cancer located in the large bowel, lung, liver, uterus and aerodigestive tract. None of these risks were statistically significant, but the small size of our study population affects the power of the study as well as the precision of the risk estimates.

A significantly higher frequency of tumor aggregations involving both first- and second-degree relatives was observed among cases as compared with controls (19 versus seven). In these families, all the affected relatives belonged to the same familial branch, and in some cases the observed aggregation partially resembled well-known cancer syndromes, although not all the established criteria for identification were met (Table 3). Although we are currently unable to establish the precise significance of this preliminary finding, it may suggest that other gene mutations (*BRCA1* and -2, *p53*, mismatch repair genes, etc.) are possibly involved and that such kindreds could therefore be usefully tested in order

to establish the proportion of pancreatic cancers attributable to different cancer syndromes.

On the basis of the distribution of age at diagnosis of pancreatic cancer in our G101W families (57.5 years) and in the present subset of apparently sporadic pancreatic cancer (60.5 years) who show a tumor clustering in the family, this feature could be reasonably hypothesized to predict positive mutation status and the melanoma–pancreas syndrome, as already reported [14, 23, 36].

p16 mutations have been found in patients with both pancreatic cancer and multiple primaries, including melanoma, as well in pancreatic cancer patients from families with pancreatic cancer and melanoma [7, 37, 38]. The two patients in our series who developed both melanoma and pancreatic cancer appeared to be carrying no *CDKN2A* mutations; however, the presence of a region of hemizyosity (D9S942 to D9S1604) including exon 1B (but not *CDKN2A*), in one of two patients points to the involvement of *p14^{ARF}* alterations in susceptibility to pancreatic cancer. Indeed, susceptibility to multiple cancers may be associated to different, contiguous tumor suppressor gene deletions, as reported for proneness to tumors of the nervous system in melanoma kindreds [27, 39].

The p16 G101W mutation recurrent in Liguria was detected in two pancreatic cancer patients out of 15 cluster families tested (13%). The presence of the conserved microsatellite core (markers D9S974 to D9S1604) confirmed the same ancestral origin previously described for this mutation in melanoma families [21]. Interestingly, the younger (48 years) of the two showed the same region of hemizyosity described above (Figure 2B and Table 4). This could point to the presence in this patient of a single G101W allele, the exon 2 mutation also affecting *p14^{ARF}*, and consequently suggest that absence of any wild-type *p14^{ARF}* may impact on pancreatic tumorigenesis. Furthermore, impairment of *p14^{ARF}* by G101W mutation translates into Arg116Cys, which in turn leads to changes in basic arginine residues within the nucleolar potential localization domain and hdm2 interaction [25].

Further characterization of the described deletion together with expression studies may further clarify whether this deletion is responsible for pancreatic cancer, or whether susceptibility to multiple cancers arises from different deletions involving tumor suppressor genes.

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