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RAPID COMMUNICATION

Lack of association between *UGT1A7*, *UGT1A9*, *ARP*, *SPINK1* and *CFTR* gene polymorphisms and pancreatic cancer in Italian patients

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Abstract

AIM: To investigate simultaneously *UGT1A7, UGT1A9, ARP, SPINK* and *CFTR* genes to verify whether genetic polymorphisms predispose to the development of pancreatic cancer (PC).

METHODS: Genomic DNA of 61 pancreatic cancer patients and 105 healthy controls (HC) were analyzed. *UGT1A7* genotyping was determined by PCR-RFLP analysis. Specific PCR and sequencing were used to analyze genetic variants of *UGT1A9, ARP, SPINK1* and *CFTR* genes.

RESULTS: Four different alleles (*1: WT; *2: N129K and R131K; *3: N129K, R131K, and W208R; and *4: W208R) in *UGT1A7* and three different alleles (*1: WT; *4: Y242X; and *5: D256N) in *UGT1A9* were detected. All UGT1A polymorphisms were observed at similar frequency in PC patients and HC. Seven different alleles in *ARP* were found in PC patients and HC at similar frequency. The *SPINK1* mutations N34S and P55S occurred in five PC patients with a prevalence (4.1%) not significantly different from that observed (2.0%) in HC. The only *CFTR* ΔF508 mutation was recognized in three PC patients with a prevalence (4.9%) similar to HC.

CONCLUSION: *UGT1A7, UGT1A9, ARP, SPINK1* and *CFTR* gene polymorphisms are not associated with PC in Italian patients.

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Key words: Pancreatic cancer; Genetic polymorphisms; Risk factors; Sporadic

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INTRODUCTION

Pancreatic cancer (PC) is still a major cause of morbidity and mortality worldwide. Despite extensive research, its etiology remains elusive. Recent data suggest that both genetic and environmental factors play a role in the development and progression of PC.

In 5%-10% of cases, PCs are inherited either as part of a known familial cancer syndrome or in association with familial forms of chronic pancreatitis[1]; in contrast, in 90%-95% of PC cases, the relationship between possible genetic predisposition and environmental risk factors is less clear. Tobacco smoking is actually recognized as the only environmental risk factor in at least 30% of all sporadic PC[2], while the role of alcohol and other xenobiotics is still controversial.

Polymorphisms in low penetrance genes involved in carcinogen metabolism and oxidative stress, such as *CYP1A1*, *CYP2D6*, *GSTM1*, *GSTT1*, *NAT1*, *UGT1A7* and *UGT1A9*, could increase susceptibility to PC through exposure to endogenous metabolites or environmental xenobiotics[3]. Interestingly, among all these detoxifying activities, the UGT1A7 and UGT1A9 seem to be the only enzymes capable of tobacco-borne toxicant inactivation[4-6]. Only few studies have investigated the polymorphism of *UGT1A7* and *UGT1A9* genes in PC patients with controversial findings[7,8].

The known pancreatic cancer susceptibility genes include germline mutations of high penetrance tumor suppressor genes or oncogenes involved in cell cycle control and DNA repair such as *CDKN2A* (also known as *p16*), *p53*, *DPC4* (also known as *SMAD4*), *STK11*, *BRCA2*,

K-ras, *hMLH1* and arginine-rich protein (*ARP*). The effect of these gene mutations in inducing PC seems to be poorly influenced by environmental factors[2]. In particular, *ARP* gene has been described as oncogene. Although the functional role of the encoded protein is still unknown, the gene has been found in all species and is highly conserved at the DNA and RNA level[9]. The presence of multiple AGG repeats around codon 50 of the mRNA and the gene has been reported in a variety of solid tumors[10,11]. Thus, variations in the ARP trinucleotide repeat region correspond to polymorphisms of the gene[12].

The known familial chronic pancreatitis susceptibility genes include germline mutations of moderate penetrance genes, such as cationic trypsinogen (*PRSS1*)[13], serine protease inhibitor Kazal type 1 (*SPINK1*)[14-16] or cystic fibrosis transmembrane conductance regulator (*CFTR*)[17] which determine hereditary pancreatitis (HP)[13], idiopathic chronic pancreatitis (ICP)[15] and cystic fibrosis (CF)[17], respectively. These genes are involved in trypsinogen activation and chloride transport, cause long-standing chronic pancreatitis and, in combination with other environmental factors such as smoking, may induce PC. However, when the polymorphisms of these genes were investigated in patients with PC, data have been inconclusive[18,19].

We hypothesize that patients with PC could have an asymptomatic chronic pancreatitis due to the interaction of environmental factors (smoking and other xenobiotics) and genetic variations of genes involved in chronic pancreatitis susceptibility or detoxifying activities (i.e. *UGT1A7*). Moreover is conceivable that these genes may interact with each other and with other known risk factors (i.e. smoking) thus increasing the risk for PC. A possible explanation of the inconclusive results of the previous studies could be the evaluation of polymorphisms of single gene. This study focused on the simultaneous investigation of the genetic variations in *UGT1A7*, *UGT1A9*, *ARP*, *SPINK1* and *CFTR* genes as potential risk factor for sporadic PC in an Italian population cohort. A possible gene-gene interaction and correlation with age at diagnosis and smoking habit was also evaluated.

MATERIALS AND METHODS

Subjects

We investigated the prevalence of *UGT1A7*, *UGT1A9*, *ARP*, *SPINK1 and CFTR* gene polymorphisms in 61 consecutive unrelated patients with sporadic pancreatic adenocarcinoma (PC) (mean age: 63 ± 10 years; range: 44-81 years; male/female: 31/30) and in 105 healthy blood donors (HC) (mean age: 39 ± 9 years; range: 19-60 years). Information on histological diagnosis and tumor location were available for all patients with pancreatic cancer (Table 1). DNA from patients and controls was obtained from peripheral blood samples after written informed consent was given. The study was approved by the Ethics Committee of our hospital.

Table 1 Clinical features of pancreatic cancer patients

Characteristic	Pancreatic cancer patients *n* = 61
Mean age ± SD (range) (yr)	63 ± 10 (44-81)
Male/Female	31 (50.8%)/30 (49.2%)
Primary tumor location	
Head	38 (62.3%)
Body	15 (24.6%)
Tail	8 (13.1%)
Smoking	
Yes/No	18 (29.5%)/43 (70.5%)

Genotyping of UGT1A7 (MIM: 606432)

UGT1A7 exon 1 polymorphic variants were identified by PCR-RFLP methods, as described by van der Logt *et al*[20]. Briefly, to detect variations at UGT1A7 codons 129/131, we used the forward primers F1 and F2 and the reverse primer R1 (Table 2). F1 only detects the N129K/R131K polymorphism, whereas F2 detects both the N129K/R131K and N129R/R131K polymorphisms. To detect the W208R alteration, we used the forward primer F3 and the reverse primer R2 (Table 2). To determine whether the N129K/R131K or N129R/R131K and W208R occur cis or trans, we used the primers F4 and R1 (Table 2). PCR conditions were 5 min at 95℃, then 37 cycles of 45 s at 95℃, 45 s at 55℃ (codons 129/131)/65℃ (codon 208) /56℃ (allele specific), and 45 s at 72℃, and finally an elongation step at 72℃ for 5 min. Aliquots of 10 μL of the PCR product were digested with the restriction enzyme *Vsp*I (codons 129/131) or *Rsa*I (codon 208/allele specific) for at least 1 h at 37℃.

Genotyping of UGT1A9 (MIM: 606434)

The strategy used to identify polymorphisms in the *UGT1A9* was PCR amplification of the exon 1, followed by direct DNA sequencing. *UGT1A9* exon 1 sequences were amplified using a 5' primer that specifically anneals to sequence upstream of the *UGT1A9* (GenBank accession number S55985) first exon, which is unique to the *UGT1A9* gene. Specific primers (Table 2) were used to amplify a PCR product of 939 bp[21]. The PCR conditions were 3 min at 94℃, then 30 cycles of 94℃ for 30 s, 57℃ for 30 s, and 72℃ for 60 s. The *UGT1A9* was sequenced on both strands for all subjects. The sequence of the PCR products was determined by automated sequencing and analysed by using Sequencing Analysis 3.4.1 (Applied Biosystems, Applera, Foster City, CA).

Genotyping of ARP (MIM: 601916)

PCR amplification of the (AGG) repeats around codon 50 of the *ARP* gene was performed using two primers, G and I (Table 2)[9], and analyzed by both sequencing and genotyping assay. For genotyping assay, forward primer was labelled with fluorescent 6-FAM dye (provided by MWG-Biotech AG). Enzymatic amplification of DNA was performed using AmpliTaq Gold® DNA Polymerase (Applied Biosystems, Applera) and amplification cycles consisted of 10 min at 94℃, followed by 35 cycles run at 94℃ for 45 s, 62℃ for 75 s and 72℃ for 45 s, and a final extension step of 72℃ for 10 min. The PCR products were analyzed for sequence by using Sequencing Analysis 3.4.1, and for genotyping by using Genescan (Applied

Table 2 Primers and restriction enzymes used for genotyping analyses

Gene	Primer (5'→3'; F = forward; R = reverse)		Restriction enzyme
UGT1A7 codon 129 and 131	F1	AAT TGC AGG AGT TTG **a**TT AA[1]	*Vsp*I
	F2	AAT TGC AGG AGT TTG **a**TT A[1]	*Vsp*I
	R1	TTC AGA GGC TAT TTC TAA GA	
UGT1A7 codon 208	F3	ATG CTC GCT GGA CGG CAC CAT TG	*Rsa*I
	R2	TGC CGT GAC AGG GGT TTG GAG A	
UGT1A7 allele specific	F4	ATT GCA GGA GTT TGT TTA AGG ACA	*Rsa*I
	R1	TTC AGA GGC TAT TTC TAA GA	
UGT1A9	F	CAGGTTTTGTGCTGGTATTTCTCCCA	
	R	GCGGATATCCATAGGCACTGGCTTTCCCTGATGACA	
ARP	G	GGCCGGGACTTGGAGGCGGTG	
	I	CAGCACGCTCAGAGCCAG	

[1]Bold 'a' in the primer sequence denotes site-directed mutagenesis for introduction of a *Vsp*I restriction site in the wild-type allele.

Biosystems, Applera, Foster City, CA).

Genotyping of SPINK1 (MIM: 167790)

The *SPINK1* polymorphisms in exon 3 were determined by automatic sequencing. PCR-RFLP (restriction endonuclease: *Taa*I, MBI Fermentas, Germany)[22] or PCR-CTPP (PCR with confronting two pair primers)[23] assays were used to confirm the N34S and P55S variants.

Genotyping of CFTR (MIM: 602421)

The 31 most frequent mutations were examined by an oligonucleotide ligation assay (OLA kit, Applied Biosystems, Applera, Foster City, CA), whereas other seven mutations, frequently observed in Italian cystic fibrosis patients were examined by sequencing as previously described[24].

Statistical analysis

The Hardy-Weinberg equilibrium was tested by using the Arlequin software[25]. Comparison of allele and genotype frequencies of *UGT1A7, UGT1A9, ARP, SPINK1* and *CFTR* polymorphism was evaluated using the Monte Carlo approach with CLUMP program[26] for the presence of multiple allele variants.

Univariate analysis was performed for tumor location, age at diagnosis and smoking habits by sorting patients according to their genotype distribution using the SPSS statistical package (SPSS v13 Inc. Chicago, IL, US). Continuous variable was expressed as means and standard deviations. Comparison of variables was performed with ANOVA method. Categorical variables were reported as percentage and comparison of them was performed by χ^2-test or Fisher exact test when appropriate.

Sample size calculation to detect a significant difference between two allelic frequencies was carried out based on data reported by Ockenga *et al*[7]. The software package available as shareware on the World Wide Web at http://statpages.org/proppowr.html was used to determine the sample size. We hypothesized an allelic frequency of 20% and 35% (difference proportion rate: 15%) in controls and pancreatic cancer patients. Setting the power of the test at 80% with a significance level of 5% and assuming a relative sample size proportion of 0.6 between pancreatic cancer patients and controls, we calculated a sample size of 198 alleles for controls and 119 alleles for patients. Consequently, we decided to recruit at least 100 controls and 60 pancreatic cancers into our study.

RESULTS

A summary of the demographic characteristics of the study population is shown in Table 1. In five subjects the diagnosis was made on clinical ground; the remaining 56 cases were confirmed histologically as ductal adenocarcinoma. In 62.3% of patients the tumor was located in the head of the pancreas, in 24.6% in the body, and in 13.1% in the tail at the time of diagnosis.

For each of the five genes, the distribution of the allele frequencies among the control population was found to fit with the assumption of the Hardy-Weinberg equilibrium ($P > 0.05$).

Three mutations (N129K, R131K, and W208R) combined into four different alleles (*1: wild type; *2: carrying N129K and R131K; *3: carrying N129K, R131K and W208R; and *4: carrying only W208R) were detected in the *UGT1A7* gene. *UGT1A7* genotyping did not show a significantly different distribution of previous alleles among patients compared to healthy controls except for the genotype *2*3 more commonly observed in healthy controls [21.0% *vs* 6.6%; CLUMP (T3): $\chi^2 = 6.05$, $P = 0.01$]. Nevertheless, after 1000 simulations with CLUMP this difference became insignificant ($Pc = 0.07$) (Table 3). Most notably, the frequency of the UGT1A7*3 risk allele was comparable between patients (42.6%) and controls (42.0%) (P = NS).

Two variations (Y242X and D256N) combined into three different alleles (*1: wild type; *4: Y242X; *5: D256N) were detected in the *UGT1A9* gene. The distribution of the UGT1A9*1, UGT1A9*4 and UGT1A9*5 alleles did not differ in PC patients and controls (Table 3). The variations were observed only in heterozygous state in both patients and controls.

In *ARP* gene a high frequency of genetic changes in the multiple arginine-coding-area such as M50R substitution (14.9% *vs* 11.1%), insertion of (AGG)n (1.4% *vs* 1.1%), deletion of (AGG)n (2.7% *vs* 4.4%) was observed in both

Table 3 Allele and genotype frequencies of *UGT1A7* and *UGT1A9* gene polymorphisms in pancreatic cancers and controls *n* (%)

Polymorphisms	Cases (*n* = 61)	Controls (*n* = 105)
***UGT1A7* (exon 1)**		
Allele frequencies		
*1	54 (44.3)	74 (35.0)
*2	15 (12.3)	46 (22.0)
*3	52 (42.6)	89 (42.0)
*4	1 (0.8)	1 (1.0)
CLUMP (T1): χ^2 = 5.66 (df = 3), *P* = 0.10		
CLUMP (T3-column used 2): χ^2 = 4.75 (df = 1), P_c = 0.07 (uncorrected *P* = 0.03)		
Genotype frequencies		
*1*1	10 (16.4)	14 (13.3)
*1*2	7 (11.5)	12 (11.4)
*2*2	2 (3.3)	6 (5.7)
*1*3	26 (42.6)	34 (32.4)
*2*3	4 (6.6)	22 (21.0)
*3*3	11 (18.0)	16 (15.2)
*1*4	1 (1.6)	0 (0.0)
*3*4	0 (0.0)	1 (1.0)
CLUMP (T1): χ^2 = 9.43 (df = 9), *P* = 0.21		
CLUMP (T3-column used 5): χ^2 = 6.05 (df = 1), P_c = 0.07 (uncorrected *P* = 0.01)		
***UGT1A9* (exon 1)**		
Allele frequencies		
*1	121 (99.2)	206 (98.0)
*5	1 (0.8)	2 (1.0)
*4	0 (0.0)	2 (1.0)
CLUMP (T1): χ^2 = 1.19 (df = 2), *P* = 0.79		
Genotype frequencies		
*1*1	60 (98.4)	101 (96.2)
*1*5	1 (1.6)	2 (1.9)
*1*4	0 (0.0)	2 (1.9)
CLUMP (T1): χ^2 = 1.19 (df = 5), *P* = 0.79		

PC and HC, respectively. Thus, we genotyped this variable site as a simple tandem repeat (STR) polymorphism. Seven different alleles (*1: 143 bp; *2: 146 bp; *3: 149 bp; *4: 152 bp; *5: 155 bp; *6: 158 bp; *7: 176 bp) were detected with wild type sequence corresponding to allele *3 and the other allele to either deletion or insertion of trinucleotide repeats. We did not observe significant difference in both allele and genotype frequencies of *ARP* between PC patients and HC (Table 4).

Direct sequencing of the coding exonic and flanking intronic sequence of the exon 3 of *SPINK1* detected mutations in 5 (4.1%) out of 61 patients. Four of them carried a heterozygous N34S mutation, and one patient had a heterozygous P55S mutation (Table 4). In 4 (2.0%) out of 105 controls two heterozygous mutations in both N34S and P55S (*P* = NS) were detected.

For the *CFTR*, only the ΔF508 mutation was found in a heterozygous state in 3 patients with PC (prevalence rate 2.5%) and in 4 controls (3.8%) (*P* = NS) (Table 4).

The statistical analysis was also performed after sorting pancreatic cancer patients out according to the histological type of the tumor, age at diagnosis and smoking habit. No statistically significant difference in genotype distribution of gene polymorphisms was observed (data no shown).

Table 4 Allele and genotype frequencies of *ARP*, *SPINK1* and *CFTR* gene polymorphisms in pancreatic cancers and controls *n* (%)

Polymorphisms	Cases (*n* = 61)	Controls (*n* = 105)
ARP		
Allele frequencies		
*1	4 (3.3)	4 (2.0)
*2	4 (3.3)	7 (3.0)
*3	111 (91.0)	195 (93.0)
*4	1 (0.8)	0 (0.0)
*5	1 (0.8)	2 (1.0)
*6	1 (0.8)	0 (0.0)
*7	0 (0.0)	2 (1.0)
CLUMP (T1): χ^2 = 5.25 (df = 6), *P* = 0.52		
Genotype frequencies		
*1*3	4 (6.6)	4 (3.8)
*2*3	4 (6.6)	7 (6.7)
*3*3	50 (82.0)	90 (85.7)
*3*4	1 (1.6)	0 (0.0)
*3*5	1 (1.6)	2 (1.9)
*3*6	1 (1.6)	0 (0.0)
*3*7	0 (0.0)	2 (1.9)
CLUMP (T1): χ^2 = 5.29 (df = 27), *P* = 0.54		
***SPINK1* (*exon* 3)**		
Allele frequencies		
wt	117 (95.9)	206 (98.0)
P55S	4 (3.3)	2 (1.0)
N34S	1 (0.8)	2 (1.0)
CLUMP (T1): χ^2 = 2.36 (df = 2), *P* = 0.29		
Genotype frequencies		
wt/wt	56 (91.8)	101 (96.2)
wt/P55S	4 (6.6)	2 (1.9)
wt/N34S	1 (1.6)	2 (1.9)
CLUMP (T1): χ^2 = 2.40 (df = 5), *P* = 0.30		
CFTR		
Allele frequencies		
wt	119 (97.5)	206 (98.0)
ΔF508	3 (2.5)	4 (2.0)
CLUMP (T1): χ^2 = 0.11 (df = 1), *P* = 1.0		
Genotype frequencies		
wt/wt	58 (95.1)	101 (96.2)
wt/ΔF508	3 (4.9)	4 (3.8)
CLUMP (T1): χ^2 = 0.11 (df = 2), *P* = 1.0		

Moreover, no evidence of interaction between investigated genes was found.

DISCUSSION

The link between genetic polymorphisms of *UGT1A*, *UGT1A9*, *ARP*, *SPINK1* and *CFTR* genes and PC represent an attractive model combining genetic predisposition with environmental exposure. In the present study, we performed a simultaneous analysis of all these five genes in patients with sporadic pancreatic carcinoma. Our working hypothesis was that the predisposition to pancreatic cancer could be due to a gene-gene interaction.

Oxidative cellular injury is a prominent mechanism that could induce pancreatic inflammation and lead to genotoxicity and cancer. A major family of proteins that

play a role in cellular detoxification and defense are the UDP-glucuronosyltransferase, which are expressed in different tissues with xenobiotic contact and have been implicated in chemical carcinogenesis[7,27]. Moreover, recent findings indicate that the presence of UGT polymorphisms may modify the risk of cancer development and reinforce the critical role of this pathway in the regulation of the biological activity of endogenous molecules and in determining the response to toxic chemicals[7]. In the present study we found that the frequency of genetic polymorphisms in *UGT1A7* in PC patients was not different from those of healthy controls. In particular, the frequency of the UGT1A7*3 allele encoding less active enzymes was 42.6% in pancreatic cancer compared to 42% in healthy controls, a figure comparable to that reported by Verlaan *et al*[8] in patients with pancreatic disease (35%-48%) and healthy controls (41%), thus suggesting that individuals bearing this allele are not at a higher risk of developing PC. Two polymorphisms of the *UGT1A9* gene (UGT1A9*4 and UGT1A9*5), described only in Japanese population[28-30], were found at a similar frequency in PC and healthy controls (0%-1%).

ARP gene has been described as a possible oncogene responsible for PC. Although the functional role of the encoded protein is still unknown, the gene was found evolutionarily conserved[9]. To examine the involvement of the *ARP* gene in exocrine PC, we screened genetic variations around multiple AGG repeats and STR polymorphism present in exon 1. We did not find a difference in mutation frequency between PC patients and controls, well in keeping with Tanaka *et al*[31], who found that the frequency for wild-type, M50R substitution, (AGG)2 insertion, and AGG deletion amounted to 82%, 14%, 3% and 1%, respectively[31]. No significant difference in allele and genotype frequencies between PC and controls for STR polymorphism were observed.

Recently, *SPINK1* polymorphisms and *CFTR* mutations have been associated with long standing chronic pancreatitis[32,33]. SPINK1 is a potent protease inhibitor that is thought to specifically inactivate intrapancreatic trypsin[34]. Evidence suggests that SPINK1 may act as a protective mechanism by preventing the activation of the pancreatic digestive enzyme cascade. Mutations in CFTR may disturb the subtle balance between proteases and their inhibitors by intrapancreatic acidification or by a defective apical trafficking of zymogen granules that might facilitate the intrapancreatic activation of digestive enzymes and result in pancreatitis[35,36]. In the current study, we observed that the prevalence of the *SPINK1* N34S and P55S polymorphisms was similar between PC patients and healthy controls as previously reported in Caucasian sporadic pancreatic cancer groups[37]. Regarding the frequency of the most common *CFTR* mutations, we found a similar prevalence of the ΔF508 mutation in PC patients and HC, consistent with similar results reported by Malats *et al*[19].

To the best of our knowledge, this is the first study to evaluate the prevalence of all five gene polymorphisms potentially involved in patients with exocrine pancreatic cancer. The findings of this study suggest that these polymorphisms are possibly not implicated in pancreatic cancer risk, although this study was powered to detect a difference proportion rate of at least 15% in allelic frequencies between controls and pancreatic cancer patients. Moreover, no gene-gene interaction or correlation with tumor location, age at diagnosis and smoking habit could be demonstrated. Further studies are needed to explore other polymorphisms involved in the metabolism of carcinogens and/or endogenous factors.

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