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Polymorphisms of *p21* and *p27* Jointly Contribute to an Earlier Age at Diagnosis of Pancreatic Cancer

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Abstract

p21 and *p27*, members of the kinase inhibitor protein (KIP) family, bind to cyclin-CDK complexes to inhibit their catalytic activity and induce cell cycle arrest. The purpose of our study was to identify whether the *p21* (C-to-A), and *p27* (T-to-G) polymorphisms were associated with age at diagnosis of pancreatic cancer, either independently or jointly. 205 patients with a diagnosis of pancreatic cancer were genotyped for the *p21* and *p27* polymorphisms. We found patients with the *p21* variant genotype (CA/AA) had an earlier age at diagnosis than those with the wild-type genotype (CC) (Log-rank, $P=0.001$; HR=1.89; 95%CI, 1.28–2.78). The *p21* and *p27* polymorphisms combined had a joint effect on age-associated risk for early diagnosis of pancreatic cancer (Log-rank, $P=0.004$; HR=2.91; 95%CI, 1.49–5.67). Our findings suggest that the *p21* polymorphism independently and *p21* and *p27* polymorphisms jointly contribute to a significantly earlier age at diagnosis of pancreatic cancer.

Keywords

p21; *p27*; polymorphisms; pancreatic cancer; age of diagnosis

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Introduction

Pancreatic cancer is the fourth leading cause of cancer death in the United States [1]. The median survival time for all patients diagnosed is less than six months with a 3% five-year survival [2]. Because early pancreatic cancer does not have any specific symptoms, tumors often go undetected until they have already spread beyond the pancreas, resulting in the worst prognosis of all solid tumors. Thus, it is of critical importance to identify biomarkers that would enable the early detection of this disease and that would allow a greater percentage of patients to undergo curative surgery.

Alterations in cell cycle regulation genes are commonly found in human neoplasia, including pancreatic cancer. Progression through the cell cycle depends on the activation of cyclins and cyclin dependent kinases (CDKs) which function together in the G1 phase for initiating S-phase and progression to G2/M phases. The activity of cyclin-CDK complexes is regulated by p21 and p27, members of the kinase inhibitor protein (KIP) family that can arrest the cell by inhibiting DNA replication [3]. p21 negatively regulates cell cycle progression by inhibiting CDK2/4 and blocks DNA replication by binding to PCNA [4]. p27 inhibits the CDK2-cyclin E complex by regulating the checkpoint at the G1 to S transition in normal cells. When p27 is absent, cells do not follow cell cycle control signs and thus proliferate in an exaggerated way [5]. The single-nucleotide polymorphisms (C-to-A) in *p21* results in an amino acid change in codon 31 from Serine to Arginine. This polymorphism is located in a highly conserved region of *p21* and is expected to affect its molecular function [6]. The single nucleotide polymorphism (T-to-G) in *p27* is located at codon 109 and results in an amino acid substitution of valine for glycine. This V109G polymorphism may have an effect on p27 degradation [7].

Given the major roles that p21 and p27 play in cell cycle checkpoint regulation, we hypothesized that polymorphisms in the two genes may interact or work together to influence the finely tuned mechanisms of cell cycle regulation. This might influence age at diagnosis of pancreatic cancer as well as the dissemination and metastasis of the disease. In this study, on a consecutive series of 205 pancreatic cancer patients, we obtained evidence that the *p21* polymorphism is associated with an earlier age at diagnosis with pancreatic cancer and that *p21* and *p27* polymorphisms jointly contribute to an earlier age at diagnosis of pancreatic cancer. We previously reported that cell cycle genes, *Aurora-A* and *p16* polymorphisms contributed to an earlier age at diagnosis of pancreatic cancer [8]. Therefore we also investigate whether these polymorphisms along with those of *p21* and *p27* jointly contribute to an earlier age at diagnosis of pancreatic cancer.

Materials and Methods

Study subjects

The study included 205 consecutively registered patients with adenocarcinoma of the pancreas evaluated at The University of Texas M. D. Anderson Cancer Center in Houston, Texas from February 1999 to October 2004. At recruitment, each participant gave written informed consent. The data that we obtained from participants included their age at diagnosis, ethnicity, and gender. The presence (M1) or absence (M0) of detectable metastases at diagnosis was determined according to the American Joint Committee on Cancer tumor-node-metastasis classification for pancreatic cancer (11). Each study subject contributed blood from which DNA was extracted with an AUTOPURE LS Automated DNA Purification Instrument (Gentra Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions. The study was approved by the Institutional Review Board of M. D. Anderson Cancer Center.

Polymorphism analysis

Genotypes of *p21* C-to-A (dbSNP: rs1801270) and *p27* G-to-T (dbSNP: rs2066827) were analyzed by pyrosequencing as according to the manufacturer's instructions (Biotage, Inc., Foxboro, MA). A polymerase chain reaction (PCR) was performed on 5 ng DNA in a 50 μ L reaction mixture containing 50 mM KCl; 10 mM Tris-HCl (pH 8.3); 2.0 mM MgCl₂; 0.125 mM dATP, dCTP, dGTP, and dTTP; 1.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems, Branchburg, NJ); and 10 pmol of each primer (Sigma/Genosys, The Woodlands, TX). The PCR reaction mixture was initially incubated at 95°C for 6 min, followed by 45 cycles at 95°C for 15 s, 63°C for 30 s for *p21* and 65°C for 30s for *p27* followed by 72°C for 15 s, and then an extension of 72°C for 5 min. The PCR primers used were 5'-CTCTTCGGCCCAGTGGACA-3' (forward) and 5'-CTCACGGGCTCCTGGAT-3' (reverse) for *p21*, 5'-CGAGTGGCAAGAGGTGGAGA-3' (forward) and 5'-GGAGCCCCAATTAAAGGCG-3' (reverse) for *p27*. The forward primer was biotinylated for *p21* and the reverse primer was biotinylated for *p27* to allow subsequent immobilization on magnetic bead. The sequencing primers were 5'-AGCGCATCACAGTCG-3' (Sigma Genosys) for *p21* and 5'-CAGGAGAGCCAGGAT-3' for *p27*. 20 samples for each polymorphism were randomly selected and repeated with 100 % concordance and the genotypes were read independently by two different persons.

The *Aurora-A* T-to-A (dbSNP: rs2273535) and *p16* C-to-T (dbSNP: rs3088440) polymorphisms were assayed as described previously [8].

Statistical analysis

We tested the association between age at diagnosis and genotype by comparing Kaplan-Meier curves according to genotype. The log-rank test was used to evaluate the homogeneity of the Kaplan-Meier curves by genotype. The Cox proportional hazard regression model was then used to estimate the association between time to diagnosis for pancreatic cancer and the polymorphic genotypes, adjusting for gender and race. We tested for Hardy-Weinberg equilibrium by using an exact test based on genotypic frequencies. The χ^2 test was used to determine the difference in the distribution of genotypes between patients with metastatic and nonmetastatic pancreatic cancer. We tested the null hypothesis for multiplicative gene-gene interaction by including main-effect variables and their product terms in the Cox regression model. A more-than-multiplicative interaction is suggested by the Hazard ratio (HR) when $HR_{11} > HR_{10} \times HR_{01}$, in which HR_{11} is the HR when both factors were present. HR_{10} is the HR when only factor 1 was present. HR_{01} is the HR when only factor 2 was present. We also tested for a more-than-additive gene-gene interaction by a bootstrapping test. A more-than-additive interaction is indicated if $HR_{11} > HR_{10} + HR_{01} - 1$. All statistical analyses were performed using the Stata 8.0 (Stata Corporation, College Station, TX).

Results

Subject characteristics

The patient characteristics are summarized in Table 1. The median age at diagnosis of the 205 patients in our study was 60.4 years (range, 25.4–80.3 years); 15.1% were ≤ 50 , 33.2% were 51–60, 29.3% were 61–70, and 22.4% were >70 years. Caucasians, Hispanics, African Americans, and Asians were 85.4%, 6.8%, 5.4%, and 2.4% of the study population, respectively. 125 (61.0%) were men and 80 (39.0%) were women. At the time of diagnosis, metastases were detected (M1) in 53 (25.9%) patients, while detectable metastases were absent (M0) in 152 (74.1%) patients. Race, sex, cigarette smoking, and history of diabetes did not show any significant effect on age at diagnosis. We genotyped the *p21* and *p27* polymorphisms in 205 pancreatic cancer patients by pyrosequencing. As shown on Table 2, the percentages in subjects were: 83.9 % (172) CC, 14.6% (30) CA and 1.5% (3) AA for

p21, and 60.0% (123) TT, 33.7% (69) TG and 6.3% (13) GG for *p27*. The genotypic frequencies for *p21* and *p27* were consistent with the Hardy-Weinberg equilibrium (for *p21*, $\chi^2 = 1.532$, $P = 0.216$; for *p27*, $\chi^2 = 0.612$, $P = 0.434$). The allelic frequencies for *p21* were C, 91%, A, 9%; for *p27* were T, 77%, G, 23%.

***p21* genotype, age at diagnosis, and cancer risk**

Because the number of subjects with the *p21* homozygous variant genotype was too low to provide meaningful results, we combined the heterozygous and homozygous variant genotypes for the analysis. Kaplan-Meier estimates showed that the median age at diagnosis in patients with the *p21* variant genotype (CA/AA) was 58, which was 3.6 years earlier than those with the *p21* wild-type genotype (CC) (Fig. 1, Table 2). The median age at diagnosis for the different genotypes was significantly different (log-rank test, $P = 0.001$). Using the subjects with the *p21* wild-type genotype as a reference in the Cox proportional hazards regression model, we found a HR of 1.89 (95% CI, 1.28–2.78) (Table 2). This result indicated that the subjects with the variant genotypes had a significantly greater probability of being diagnosed with pancreatic cancer during any interval than those with the wild-type genotype.

***p27* genotype, age at diagnosis, and cancer risk**

Because of low frequency of homozygous variant carriers, we combined the heterozygous and homozygous variant genotypes for the analysis. The median ages of diagnosis for the different genotypes were determined from Kaplan-Meier plots and are shown in Table 2. There was no significant difference in age at diagnosis between the variant genotype (TG/GG) and wild-type genotype (TT) for the *p27* polymorphism (Log-rank test, $P = 0.536$). Compared to patients carrying wild-type genotype, patients with variant genotypes did not show a significant increase in the HR (1.09; 95% CI, 0.82–1.44).

Combined effect and pancreatic cancer risk

Because both *p21* and *p27* are involved in the same cell cycle regulation pathway, we evaluated the effect of combined genotypes upon time-to-diagnosis of cancer. We found that patients with variant genotypes of both *p21* and *p27* were diagnosed with pancreatic cancer 8.6 years earlier than those with both wild-type genotypes. The median ages at diagnosis for patients with zero, one or two variant genotypes were 61.4, 60.4, and 52.8 years, respectively. Kaplan-Meier plots (Fig. 2) showed a significant difference in age at diagnosis among the different genotypes (Log-rank test, $P = 0.004$). We also determined whether the *p21* and *p27* polymorphisms had a joint effect on the age-associated risk of pancreatic cancer. We found that patients with a variant genotype for only one of the two genes had a slightly higher nonsignificant age-associated risk for diagnosis of pancreatic cancer than patients with wild-type genotypes for both genes (HR = 1.20; 95% CI, 0.90–1.60 Table 2). Interestingly, the HR increased to 2.91 (95% CI, 1.49–5.67) for patients carrying variant genotypes for both genes, and these results were confirmed using bootstrapping which showed a 95% CI of 1.66–5.08. These results indicate a joint effect between the *p21* variant genotypes and the *p27* variant genotypes on the age-associated risk for diagnosis of pancreatic cancer. The test for multiplicative gene-gene interaction was close to significant ($P = 0.080$). A test of an additive model performed by bootstrapping showed significant departure from an additive model ($P = 0.012$), further supporting a joint effect when both risk genotypes were present.

Because we previously reported that *Aurora-A* and *p16* polymorphisms also contributed to an earlier age at diagnosis of pancreatic cancer, we evaluated the combined effect of polymorphisms of the four cell cycle genes upon time-to-diagnosis of cancer. We found that patients with 3–4 variant genotypes were diagnosed with pancreatic cancer 12.1 years earlier

than those with zero variant genotype. The median ages at diagnosis for patients with zero, 1–2 variant genotypes, and 3–4 variant genotypes were 65.7, 59.8, and 53.6 years, respectively. Kaplan-Meier plots (Fig. 3) showed a significant difference in age at diagnosis among the different genotypes (Log-rank test, $P = 0.0004$). Compared with the reference group (zero variant genotype), patients with 1–2 variant had a slightly higher age-associated risk for diagnosis of pancreatic cancer (HR = 1.47; 95% CI, 1.07–2.02, while the HR increased to 3.36 (95% CI, 1.76–6.40) for patients carrying 3–4 variant genotypes (table 3).

The effect of *p21* and *p27* polymorphisms on cancer status

We also determined the potential effect of the *p21* genotype on tumor invasion and metastasis. No significant differences in the *p21* genotypic frequencies between subgroup M1 and subgroup M0 were observed ($\chi^2 = 0.167$; $P = 0.683$). Additionally, the variant genotype was not significantly associated with survival after diagnosis (Log-rank test, $P = 0.830$). Similarly, no significant differences in the *p27* genotypic frequencies between subgroup M1 and subgroup M0 were observed (data not shown). Furthermore, no significant association between the polymorphism and disease status was found when patients in stage I were compared with patients in stage II – IV for *p21* and *p27* (data not shown).

Discussion

In this study, we found that *p21* polymorphism contributes to a significantly earlier age at diagnosis of pancreatic cancer and that *p21* and *p27* polymorphisms cooperatively contribute to an earlier age at diagnosis of pancreatic cancer. Patients with one or two variant alleles in both genes had a 2.91-fold increased risk for earlier age at diagnosis of pancreatic cancer compared with wild-type genotypes and were diagnosed with pancreatic cancer 8.6 years earlier. These observations indicate that the polymorphisms of these two genes could be risk markers for early detection of pancreatic cancer.

When the functional *p21* polymorphism was analyzed alone for influence on age at diagnosis of pancreatic cancer, patients with the variant genotypes (CA/AA) developed pancreatic cancer earlier than did those with the wild-type genotype (CC) and had an approximately 1.89-fold increased age-associated risk. It is biologically plausible that the *p21* polymorphism might influence age onset of cancer, as p21 can arrest the cell cycle in G1 phase, in order to allow repair of damaged DNA and inhibition of carcinogenesis [9]. Current studies suggest that the polymorphism is likely to contribute to genetic susceptibility to cancer [10], [11], [12], [13], [14] and [15], although some studies found no association between the polymorphism and cancer risk, including lung cancer [16] and [17] and breast cancer [18]. This discrepancy could be due to the different carcinogenic mechanisms (different cancers), underlying genetic backgrounds or environmental and social factors in the different populations studied. Another possible explanation is that the polymorphism is in linkage disequilibrium with other functional variants at susceptibility loci. However, this hypothesis remains to be tested.

There was no significant difference in age at diagnosis between the variant genotype (TG/GG) and wild-type genotype (TT) for the *p27* polymorphism. Patients with variant genotypes did not show a significantly higher age-associated risk than those who carried wild-type genotype. Our result is consistent with other studies that did not observe any association with cancer risk [18], [19] and [20]. However, the *p21* and *p27* polymorphisms combined had a joint effect on age-associated risk for early diagnosis of pancreatic cancer. Compared to patients with wild-type genotypes for both genes, the median age at diagnosis for patients with one or two variant alleles for both genes was 8.6 years earlier. We found that patients with a variant genotype for only one of the two genes had a slightly higher nonsignificant age-associated risk for diagnosis of pancreatic cancer than patients with wild-

type genotypes for both genes (HR = 1.20; 95% CI, 0.90–1.60). Interestingly, the HR increased to 2.91 (95% CI, 1.49–5.67) for patients carrying variant genotypes for both genes. These results indicate a joint effect between the *p21*/variant genotypes and *p27* variant genotypes on the age-associated risk for diagnosis of pancreatic cancer. A test of an additive model performed by bootstrapping showed significant departure from an additive model ($P = 0.012$), further supporting a joint effect when both risk genotypes were present.

p21 and *p27*, members of the KIP family, inhibit the activities of cyclin D-, E-, and A-dependent kinases and induce cell-cycle arrest to allow cells to stop and repair DNA lesions before DNA replication occurs in S phase. Low levels of *p27* are reported to be causally related to tumorigenesis [21]. The *p21* C-to-A polymorphism in codon 31 causing a serine-to-arginine substitution in the DNA-binding zinc-finger motif could encode functionally distinct proteins [22] and [23]. The *p27* T-to-G polymorphism in codon 109 causing a valine-to-glycine substitution may affect the interaction between *p21* and its negative regulator *p38* and thereby modify *p21* degradation [24]. Since both the *p21* and *p27* polymorphisms are low penetrance mutations that only slightly alter protein functions, the combined effects of the two polymorphisms could be to intensify cell cycle arrest after DNA damage and lead to incompletely repaired genomes, thereby suppressing the apoptosis response. Future studies need to clarify the functional effects of the *p21* and *p27* polymorphisms.

In order to evaluate aggregate effect of polymorphisms in cell cycle regulator genes, we performed a combined variant genotype analysis that include *Aurora-A* and *p16* polymorphisms which we previously reported [8]. We found that patients with 3–4 variant genotypes were diagnosed with pancreatic cancer 12.1 years earlier than those with zero variant genotype (Log-rank test, $P = 0.0004$). Compared with the reference group (zero variant genotype), patients with 1–2 variant had a slightly higher age-associated risk for diagnosis of pancreatic cancer (HR = 1.47; 95% CI, 1.07–2.02), while the HR increased to 3.36 (95% CI, 1.76–6.40) for patients carrying 3–4 variant genotypes. Our finding suggests that combining multiple polymorphisms in a single pathway (the cell cycle pathway) may be powerful in identifying patients at increased risk for developing pancreatic cancer at an early age.

Our data did not show significant correlation between the polymorphisms and the cancer's metastatic status or stage. That may be because the development of pancreatic cancer is a multistep process with many factors contributing to the pathogenesis and progression. Those polymorphisms may not influence later events since other factors may begin to have greater influence as the cancer begins to metastasize.

Because early pancreatic cancer does not have any specific symptoms, tumors often go undetected until they have already spread beyond the pancreas, resulting in the worst prognosis of all solid tumors. New approaches to earlier diagnosis are important for improving the prognosis of the disease. If confirmed, the present findings, combined with the identification of additional environmental and genetic risk factors, could provide a panel of risk markers, which could help identify those patients who are more likely to develop pancreatic cancer and this could lead to earlier detection and treatment, longer survival time, and lower mortality of pancreatic cancer. Future studies will focus on identifying additional risk factors.

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Fig. 1. Kaplan-Meier curves showing cumulative risk for diagnosis of pancreatic cancer by patient age for the CA/AA and CC genotypes of the *p21* polymorphism.

Fig. 2.

Kaplan-Meier curves showing cumulative risk for diagnosis of pancreatic cancer by patient age for the combined effect of *p21* and *p27* polymorphisms. The patients have either zero (both genes are wild-type), one (only one of two genes is variant), or two (both genes are variant) variant genotypes.

Fig. 3. Kaplan-Meier curves showing cumulative risk for diagnosis of pancreatic cancer by patient age for the combined effect of variant genotypes. The patients have zero variant genotype, 1–2 variant genotypes, or 3–4 variant genotypes.

Table 1

Patient Characteristics

Variable	No. patients	No. deaths
Age at Diagnosis (years)		
50	31	27
51–60	68	55
61–70	60	47
>70	46	38
Sex		
Male	125	106
Female	80	61
Race		
Caucasian	175	142
Hispanics	14	12
African American	11	9
Asian	5	4
Smoking		
Never	65	49
Ever	140	118
Diabetes		
No	154	121
Yes	51	46
Metastases		
No	152	116
Yes	53	49

Table 2Genotypes of *p21* and *p27* and their association with risk of pancreatic cancer

Genotypes	(N = 205)	Median age	HR [*]	P value [†]
	No. (%)	at diagnosis	(95% CI)	
p21 (C-to-A)				
CC	172 (83.9)	61.6	1.00 (reference)	0.001
CA+AA	33 (16.1)	58.0	1.89 (1.28–2.78)	
p27 (T-to-G)				
TT	123 (60)	60.3	1.00 (reference)	0.536
TG+GG	82 (40)	60.2	1.09 (0.82–1.44)	
Combined				
0 variant genotype	100 (48.8)	61.4	1.00 (reference)	0.004
1 variant genotypes	95 (46.3)	60.4	1.20 (0.90–1.60)	
2 variant genotypes	10 (4.9)	52.8	2.91 (1.49–5.67)	

* HR, hazard ratios and CI, confidence intervals.

† Log-rank test for homogeneity between genotypes.

Table 3

Joint effects of variant genotypes and their association with risk of pancreatic cancer

No. of variant genotypes	(N = 205)	Median age	HR [*]	P value [†]
	No. (%)	at diagnosis	(95% CI)	
0 variant genotype	57 (27.8)	65.7	1.00 (reference)	0.0004
1–2 variant genotypes	136 (66.3)	59.8	1.47 (1.07–2.02)	
3–4 variant genotypes	12 (5.9)	53.6	3.36 (1.76–6.40)	

^{*} HR, hazard ratios and CI, confidence intervals.

[†] Log-rank test for homogeneity between genotypes.