Published in final edited form as:
Clin Cancer Res. 2007 May 15; 13(10): 3100–3104.

Aurora-A and *p16* Polymorphisms Contribute to an EarlierAge at Diagnosis of Pancreatic Cancer in Caucasians

Jinyun Chen[1], **Donghui Li**[2], **Chongjuan Wei**[1], **Subrata Sen**[3,6], **Ann M. Killary**[4,6], **Christopher I. Amos**[1,6], **Douglas B. Evans**[5], **James L. Abbruzzese**[2], and **Marsha L. Frazier**[1,6]

1 *Department of Epidemiology, The University of Texas, Houston, Texas*

2 *Department of Gastrointestinal Medical Oncology, The University of Texas, Houston, Texas*

3 *Department of Molecular Pathology, The University of Texas, Houston, Texas*

4 *Department of Cancer Genetics, The University of Texas, Houston, Texas*

5 *Department of Surgical Oncology, M. D. Anderson Cancer Center, The University of Texas, Houston, Texas*

6 *Program in Human and Molecular Genetics, Graduate School of Biomedical Sciences, The University of Texas, Houston, Texas*

Abstract

Purpose—Aurora-A and p16 play a major role in cell cycle checkpoint regulation. Both of them are important in the maintenance of centrosome duplication. Therefore, we hypothesized that polymorphisms in the two genes may interact or work together to influence the finely tuned mechanisms of cell cycle regulation that these proteins regulate. The purpose of this study was to investigate the association of the *Aurora-A* (T91A), and *p16* (C540G and C580T) polymorphisms with age at diagnosis of pancreatic cancer.

Experimental Design—We genotyped 148 Caucasian patients with a diagnosis of pancreatic cancer for the *Aurora-A* and *p16* polymorphisms using pyrosequencing. We tested the association between age at diagnosis and the *Aurora-A* and *p16* genotypes by comparing Kaplan-Meier curves, evaluating the homogeneity of the curves using the log-rank test. We used Cox proportional hazard regression analysis to estimate the association between time to diagnosis and genotype, adjusting for gender.

Results—Patients with the *Aurora-A* polymorphic genotypes had a median age at diagnosis with pancreatic cancer that was 2.8 years earlier than those with the wild-type genotype [log-rank, P = 0.015; hazard ratio (HR), 1.55; 95% confidence intervals (95% CI), 1.09–2.20]. There was no significant association between the *p16* genotypes and age at diagnosis. However, the *Aurora-A* and *p16* C580T polymorphisms combined had a synergistic effect on age-associated risk for early diagnosis of pancreatic cancer. Compared with patients with wild-type genotypes for both genes, the median age at diagnosis for patients with one or two polymorphic alleles for both genes was 12.6 years earlier (log-rank, P = 0.0002; HR, 3.88; 95% CI, 1.94–7.76). No significant associations between the polymorphisms and the cancer metastatic status or survival after diagnosis were found.

Conclusions—Our findings suggest that the *Aurora-A* polymorphism contributes to a significantly earlier age at diagnosis of pancreatic cancer, and that *Aurora-A* and *p16* C580T polymorphisms synergistically contribute to an earlier age at diagnosis of pancreatic cancer.

Requests for reprints: Marsha L. Frazier, Department of Epidemiology, Unit1365, The University of Texas M. D. Anderson Cancer Center, 1155 Pressler Boulevard, Houston, TX 77030. Phone: 713-792-3393; Fax: 713-563-0999; E-mail: mlfrazier@mail.mdanderson.org.

Pancreatic cancer is the fourth leading cause of cancer-related mortality in the United States, with 33,370 deaths predicted to occur from the disease in 2007 (1). Because of the asymptomatic onset of pancreatic cancer, most patients already have metastatic or locally advanced disease at the time of diagnosis, resulting in poor prognoses. Thus, novel approaches leading to the earlier diagnosis and treatment of this deadly disease are needed. More importantly, identifying the genetic risk factors associated with pancreatic cancer could lead to strategies to prevent it.

Uncontrolled cell proliferation is a characteristic of tumor cells, and mutations in the genes involved in cell cycle control are frequent in human cancers, suggesting that the inactivation of their pathways may be necessary for tumor development (2). Aurora-A (also known as STK15, BTAK, AIKI, and AURKA) is a serine/threonine kinase and plays a pivotal role in proper mitotic entry and the G_2-M checkpoint. The overexpression of Aurora-A induces abnormal G_2-M transition in mammalian cells and may lead to centrosome amplification and chromosome instability, which results in the development and progression of malignant tumors (3,4). A recent study showed that Aurora-A is overexpressed in pancreatic tumors and carcinoma cell lines, suggesting that its overexpression plays a role in pancreatic carcinogenesis (5). A common T-A polymorphism (T91A) has been identified at codon 31 of the *Aurora-A* gene, resulting in a phenylalanine to isoleucine substitution. The A allele has been reported to be preferentially amplified and associated with the degree of aneuploidy in human tumors (6).

p16 (also known as CDKN2, MTS-1, and INK4a) plays a pivotal role in the regulation of the G_1-S cell cycle checkpoint. It blocks cell cycle progression by binding CDK4/6 and inhibiting the action of D-type cyclins (7). This leads to accumulation of hypophosphorylated Rb and growth arrest in G_1 (8). Germ line mutations in the *p16* gene are known to predispose individuals to pancreatic cancer and melanoma (9). Two adjacent polymorphisms in the *p16* gene (C540G and C580T) located in the 3′untranslated region of exon 3 are associated with a significantly shorter progression time from primary to metastatic melanoma (10).

Given the major roles that Aurora-A and p16 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 148 Caucasian pancreatic cancer patients, we obtained evidence that the *Aurora-A* T91A polymorphism is associated with an earlier age at diagnosis with pancreatic cancer, and that *Aurora-A* and *p16* C580T polymorphisms synergistically contribute to an earlier age at diagnosis of pancreatic cancer.

Materials and Methods

Study subjects

The study included 148 consecutively registered Caucasian patients with adenocarcinoma of the pancreas evaluated at the University of Texas M. D. Anderson Cancer Center in Houston, Texas, from February 1999 to August 2004. At recruitment, each participant gave written informed consent. The data that we obtained from participants included their age at diagnosis and gender. The presence (M_1) or absence (M_0) 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.) 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 *Aurora-A* T91A (dbSNP: rs2273535), *p16* C540G (dbSNP: rs11515), and *p16* C580T (dbSNP: rs3088440) were analyzed by pyrosequencing as directed by the manufacturer (Biotage, Inc.). A PCR was done on 5 ng DNA in a 50-μL reaction mixture containing 50 mmol/L KCl; 10 mmol/L Tris-HCl (pH, 8.3); 2.0 mmol/L $MgCl_2$; 0.125 mmol/L dATP, dCTP, dGTP, and dTTP; 1.5 units AmpliTaq Gold DNA polymerase (Applied Biosystems); and 10 pmol of each primer (Sigma/Genosys). The PCR reaction mixture was initially incubated at 95°C for 6 min, followed by 45 cycles at 95°C for 15 s, 64°C for 30 s for Aurora-A, and 67°C for 30 s for p16, followed by 72°C for 15 s, and then an extension of 72°C for 5 min. The PCR primers used were 5′-CCATTCTAGGCTACAGCTCCA-3′ (forward) and 5′-ATTCTGAACCGGCTTGTGAC-3′ (reverse) for *Aurora-A*, 5′-GTGCCACACATCTTTGACCTCAG-3′ (forward) and 5′-TACGAAA-GCGGGGTGGGT-3′ (reverse) for *p16* (C540G and C580T). The reverse primer was biotinylated for *Aurora-A*, and the forward primer was biotinylated for *p16* to allow subsequent immobilization on a magnetic bead. The sequencing primers were 5′-TCTCGTGACTCAGCAA-3′ (Sigma Genosys) for *Aurora-A*, 5′-GACTGATGATCTAAGTTTCC-3′ for *p16* C540G, and 5′-TGTGGCGGGGGCAGT-3′ for *p16* C580T. Sixteen samples for each polymorphism were randomly selected and repeated with 100% concordance, and the genotypes were read independently by two different persons.

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. We tested for Hardy-Weinberg equilibrium by using an exact test based on genotypic frequencies (12). We used the LDA software to calculate the linkage disequilibrium index (13). Haplotype associations with the genotypes and age at diagnosis of pancreatic cancer were calculated with the haplotype procedure in the SAS/Genetics module (SAS, version 9.1). The χ^2 test was used to determine the difference in the distribution of genotypes between patients with metastatic and nonmetastatic pancreatic cancer. The association between the polymorphic genotypes and the risk of metastatic pancreatic cancer was estimated by odds ratios (OR) and 95% confidence intervals (95% CI), which were calculated by unconditional logistic regression models. The ORs were adjusted for age and gender. We tested the null hypotheses of 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). $HR_{11} > HR_{10} \times HR_{01}$, 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 done using the Stata 8.0 (Stata Corporation).

Results

Subject characteristics

Of the 148 Caucasian patients in our study, 82 (55.4%) were men, and 66 (44.6%) were women. At the time of diagnosis, metastases were detected (M_1) in 39 (26.4%) patients, whereas detectable metastases were absent (M_0) in 109 (73.6%) patients. We genotyped the *Aurora-A* and *p16* polymorphisms in 148 pancreatic cancer patients by pyrosequencing. The percentages in subjects were, for *Aurora-A*, 68.2% (101) TT, 27.1% (40) TA, and 4.7% (7) AA; for p*16* C540G, 72.3% (107) CC, 25.7% (38) CG, and 2.0% (3) GG; for *p16* C580T,

81.1% (120) CC, 16.9% (25) CT, and 2.0% (3) TT. The genotypic frequencies for *Aurora-A* and *p16* were consistent with the Hardy-Weinberg equilibrium (for *Aurora-A*, $\chi^2 = 1.307$, $P = 0.253$; for *p16* C540G, $\chi^2 = 0.031$, $P = 0.861$; for *p16* C580T, $\chi^2 = 1.457$, $P = 0.228$). The allelic frequencies for *Aurora-A* are T, 81.8%, A, 18.2%; for *p16* C540G, C, 85.1% and G, 14.9%; and for *p16* C580T, C, 89.5%, T, 10.5%.

Aurora-A genotype, age at diagnosis, and cancer risk

Because the number of subjects with the *Aurora-A* homozygous polymorphic genotype was too low to provide meaningful results, we combined the heterozygous and homozygous polymorphic genotypes for the analysis. Kaplan-Meier estimates showed that the median age at diagnosis in patients with the *Aurora-A* polymorphic genotype was 59.6 years, which was 2.8 years earlier than those with the *Aurora-A* wild-type genotype (Fig. 1). The median age at diagnosis for the different genotypes was significantly different (log-rank test, $P = 0.015$). Using the subjects with the *Aurora-A* wild-type genotype as a reference in the Cox proportional hazards regression model, we found a HR of 1.55 (95% CI, 1.09–2.20; Table 1). This result indicated that the subjects with the polymorphic genotypes had a significantly greater probability of being diagnosed with pancreatic cancer during any interval than those with the wild-type genotype.

p16 genotype, haplotype, age at diagnosis, and cancer risk

Because of low frequency of homozygous polymorphic carriers, we combined the heterozygous and homozygous polymorphic genotypes for the analysis. The median ages of diagnosis for the different genotypes were determined from Kaplan-Meier plots and are shown in Table 1. There was no significant difference in age at diagnosis between the polymorphic genotype and wild-type genotype for C540G (log-rank test, $P = 0.252$) and a borderline significant difference between polymorphic genotypes and wild-type genotypes for C580T (log-rank test, $P = 0.050$). Compared with patients carrying wild-type genotype, patients with polymorphic genotypes of C540G did not show a significant increase in the HR (0.80; 95% CI, 0.56–1.17), and C580T showed a borderline significant increase in the HR (1.52; 95% CI, 1.00–2.30) in age-associated risk for the diagnosis of pancreatic cancer. Significant linkage disequilibrium (LD) was found between the two adjacent polymorphisms ($D' = 1$, $P = 0.025$). Using the expectation-maximization (EM) algorithm to estimate the frequencies of the haplotypes, there were three of four possible haplotypes derived from the known genotypes (540C–580C, 74.7%; 540G–580C, 14.8%; and 540C–580T, 10.5%). The frequency of 540G–580T haplotype was zero. Therefore, only six diplotypes were determined in this study population. Most common diplotypes were 540C–580C/540C–580C (83, 56.1%), 540C–580C/540G–580C (34, 23.0%), and 540C–580C/540C–580T (21, 14.2%). Although 3 (2.0%) were 540G–580C/540G–580C, 4 (2.7%) were 540G–580C/540C–580T, and 3 (2.0%) were 540C–580T/540C–580T. No difference was observed in age-associated risk for the diagnosis of pancreatic cancer among diplotypes (data not shown).

Gene-gene interaction and pancreatic cancer risk

We evaluated the effect that carriage of multiple polymorphic alleles had on time to diagnosis of cancer. Interestingly, we found that patients with polymorphic genotypes of both *Aurora-A* and *p16* C580T were diagnosed with pancreatic cancer 12.6 years earlier than those with both wild-type genotypes. The median ages at diagnosis for patients with zero, one, or two polymorphic genotypes were 63.4, 60.3, and 50.8 years, respectively. Kaplan-Meier plots (Fig. 2) showed a significant difference in age at diagnosis among the different genotypes (log-rank test, $P = 0.0002$). To determine if the earlier median age at diagnosis among the patients with two polymorphic genotypes might be due to familial pancreatic cancer, we assessed family history. None of the patients had a history of pancreatic cancer and/or melanoma. Although

seven patients were homozygous for the rare allele of the *Aurora-A* gene and three patients were homozygous for the rare allele of the *p16* gene, none of the subjects were homozygous for the rare allele for both *p16* and *Aurora-A*. The expected frequency of homozygosity for the rare allele of the *Aurora-A* gene is 3.3% (we observed 4.7%) and, for *p16* gene, 1.1% (we observed 2%). The probability of homozygosity for the rare allele for both *p16* and *Aurora-A* is very low ($P = 0.0004$). Therefore, it is not surprising that we did not observe this genotype in our subjects.

We also determined whether the *Aurora-A* and *p16* (C580T) polymorphisms had a joint effect on the age-associated risk of pancreatic cancer. We found that patients with a polymorphic 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, 1.29; 95% CI, 0.77–2.15 for *p16*; HR, 1.41; 95% CI, 0.95–2.10 for *Aurora A*; Table 2). Interestingly, the HR increased to 3.88 (95% CI, 1.94–7.76) for patients carrying polymorphic genotypes for both genes (log-rank test, $P = 0.0006$), and these results were confirmed using bootstrapping, which showed a 95% CI of 2.2–6.8. These results indicate a more-than-multiplicative joint effect between the *Aurora-A* polymorphic genotypes and *p16* (C580T) polymorphic genotypes on the age-associated risk for diagnosis of pancreatic cancer. For example, the product of the separate effects is $1.29 \times 1.41 = 1.82$ (i.e., a multiplicative effect), which is much smaller than the observed combined effect of 3.88 (Table 2). However, the test for multiplicative gene-gene interaction was not quite significant ($P = 0.091$). A test of an additive model done by bootstrapping showed significant departure from an additive model ($P = 0.029$), further supporting multiplicative or synergistic effect when both risk genotypes were present.

We also evaluated the effect that carriage of multiple polymorphic alleles of the *Aurora-A* and *p16* (C540G) had. No joint effect on the age-associated risk of pancreatic cancer was observed (data not shown).

The effect of *Aurora-A* and *p16* polymorphisms on cancer status

We also determined the potential effect of the *Aurora-A* genotype on tumor invasion and metastasis. No significant differences in the *Aurora-A* genotypic frequencies between subgroup M_1 and subgroup M_0 were observed ($\chi^2 = 0.419$; $P = 0.517$). Additionally, the polymorphic genotype was not significantly associated with the risk of metastatic disease compared with the wild-type genotype (OR, 1.29; 95% CI, 0.60–2.77) or survival after diagnosis (log-rank test, $P = 0.786$). Similarly, no significant differences in the *p16* genotypic frequencies between subgroup M_1 and subgroup M_0 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 stages II to IV for *Aurora-A* and *p16* (data not shown).

Discussion

In this study, we examined polymorphisms in two cell cycle genes, *Aurora-A* and *p16.* We found that *Aurora-A* T91A and *p16* C580T polymorphisms had a synergistic effect on age-associated risk for diagnosis of pancreatic cancer. Patients with one or two polymorphic alleles in both genes had a 3.88-fold increased risk for earlier age at diagnosis of pancreatic cancer compared with wild-type genotypes and were diagnosed with pancreatic cancer 12.6 years earlier. This is the first report, to our knowledge, to show that the *Aurora-A* and *p16* polymorphisms synergistically contribute to an earlier age at diagnosis for any cancer.

When the functional *Aurora-A* T91A polymorphism was analyzed alone for influence on age at diagnosis of pancreatic cancer, patients with the polymorphic genotypes developed pancreatic cancer earlier than did those with the wild-type genotype and had an approximately

1.55-fold increased age-associated risk. These findings are consistent with those of other studies, which show the A allele to be an adverse genotype (14–16). Ewart-Toland et al. (17) also reported that the A allele increased the risk of multiple cancer types and confirmed that the allele is a low-penetrance cancer susceptibility allele by carrying out a meta-analysis. In contrast, other recent studies reported that the A allele was associated with a significantly reduced risk for lung cancer in Caucasians (18), and no association between the A allele and the risk for breast cancer was observed in English women (19).

No significant association was observed between *p16* C540G or *p16* C580T genotypes alone and age at diagnosis with pancreatic cancer. Although the two adjacent polymorphisms of *p16* were reportedly associated with tumor aggressiveness for melanoma (10), Zheng et al. (20) did not find that they were associated with the risk of developing squamous cell carcinoma of the head and neck.

The mechanism for the synergistic effect between *Aurora-A* T91A and *p16* C580T upon age at diagnosis is unknown. It may be that there are physical interactions between the two genes that are influenced by the polymorphisms or the polymorphisms may influence the physical interactions of Aurora-A and p16 with other proteins or with environmental factors such as tobacco smoke. The overexpression of Aurora-A has been shown to lead to centrosome amplification, chromosomal instability, and transformation in mammalian cells (4). Aurora-A localizes to centrosomes immediately after the centrioles have been duplicated at the end of S phase and becomes phosphorylated and activated in centrosomes late in the G2 phase. The protein then positively regulates the G2-M phase transition of the cell cycle. p16 has also been shown to be important in proper centrosome duplication. Loss of p16 results in generation of supernumerary centrosomes in studies on primary diploid epithelial and fibroblast cultures (21). The synergism between Aurora-A and p16 could be a result of subtle variations caused by polymorphisms in the two genes, which influence their ability to carry out their roles in proper maintenance and duplication of the centrosomes.

Another possible mechanism by which Aurora-A and p16 could have a synergistic effect on age at diagnosis of pancreatic cancer is through their influence on the G_1-S checkpoint. p16 is important in the regulation of the G_1-S checkpoint as it binds to CDK4 and CDK6, inhibiting the ability of either protein to interact with cyclin D1 and stimulate passage through the G_1-S phase transition of the cell cycle. Although Aurora-A is important in mitotic entry and G_2-M checkpoint, it may also play a role in the G_1-S checkpoint because Aurora-A kinase activity has been shown to modulate p53 stability. p53 is important in several functions, including regulation of the G_1-S checkpoint where it positively regulates p21, in response to DNA damage, which then binds to cyclin D1/CDK4 or CDK6 complexes to inhibit their activity and induce cell cycle arrest (22,23). The synergism might therefore arise as a result of the combined influence that Aurora-A and p16 have on the G_1-S cell cycle checkpoints.

Further confirmation of the synergism between the *p16* and *Aurora-A* polymorphisms in additional clinical populations and a better understanding of the mechanism by which this synergism occurs are needed.

Several studies have shown that the overexpression of Aurora-A is more relevant for early events in tumorigenesis than for tumor progression (5,24–26). Our findings are in agreement with these previous studies because the *Aurora-A* polymorphism was significantly correlated with the age at diagnosis of pancreatic cancer but not with its progression, as shown by the lack of significant correlation between the polymorphism and the cancer's metastatic status or stage. Thus, our findings agree with previous studies and further substantiate that *Aurora-A* is a potential low-penetrance cancer susceptibility gene in humans (6).

Because pancreatic cancer is usually not detected until it reaches an advanced stage, 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 the earlier detection and treatment, longer survival time, and lower mortality of pancreatic cancer. Future studies will focus on identifying additional risk factors.

Acknowledgements

We thank Haidee Chancoco for DNA extraction, and Christopher Yeager, Angelique Siy, and Monica Domingue for their editorial comments.

Grant support: National Cancer Institute grants P20 CA101936, and U01 CA111302; NIHCancer Center Support grant CA16672.

This research was supported, in part, by the Janis Davis Gordon Memorial Postdoctoral Fellowship, Division of Cancer Prevention, University of Texas M. D. Anderson Cancer Center.

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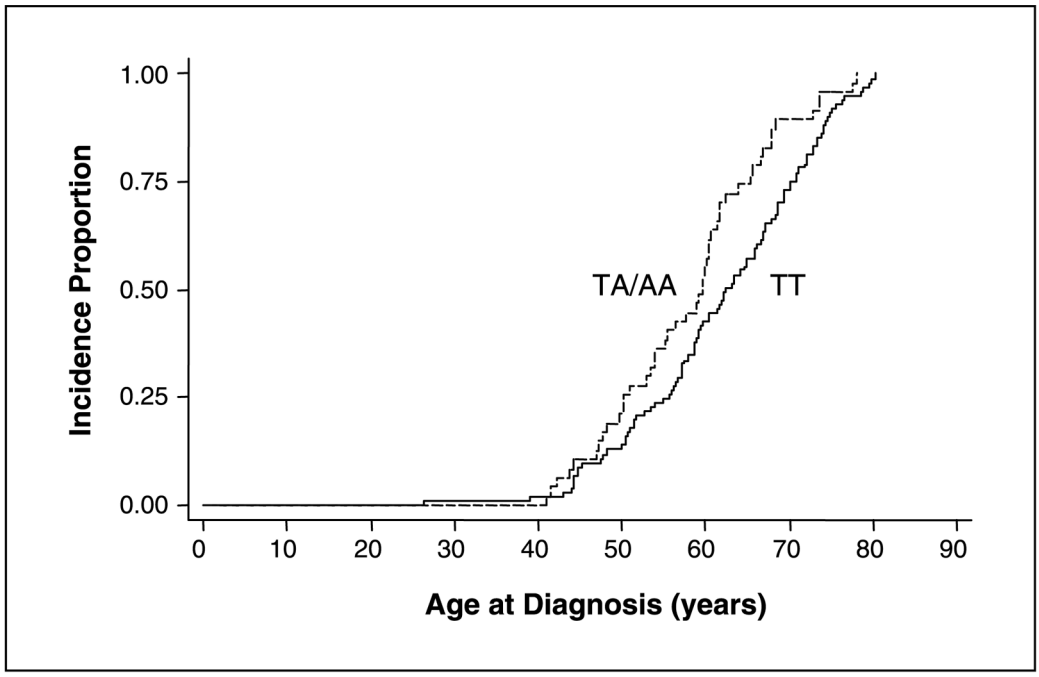

Fig. 1.
Kaplan-Meier curves showing cumulative risk for diagnosis of pancreatic cancer by patient age for the TA/AA andTTgenotypes of the *Aurora-A* polymorphism.

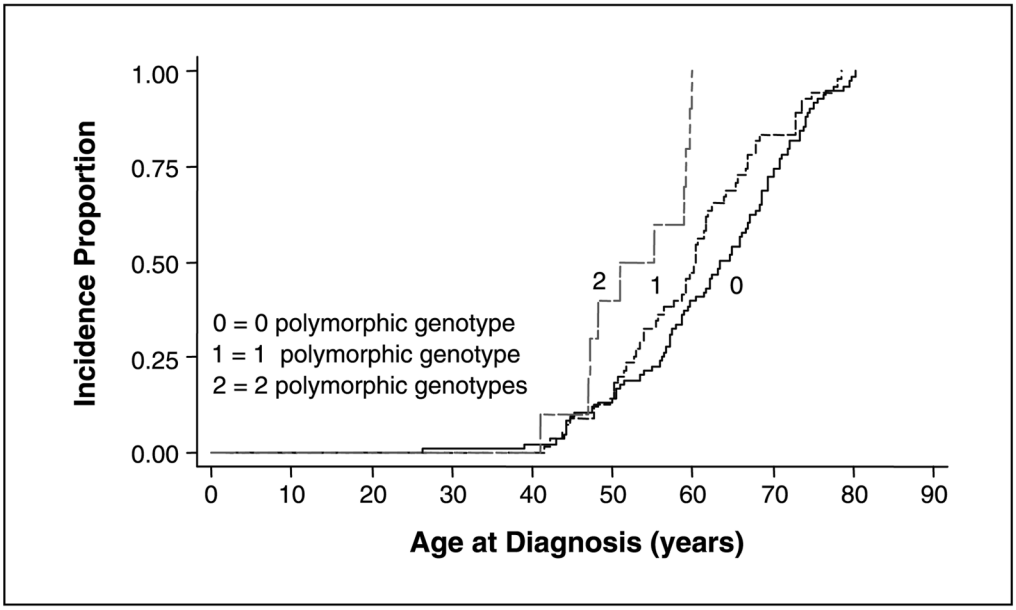

Fig. 2.

Kaplan-Meier curves showing cumulative risk for diagnosis of pancreatic cancer by patient age for the combined effect of *Aurora-A* and *p16* C580T polymorphisms. The patients have either zero (both genes are wild type), one (only one of two genes is polymorphic), or two (both genes are polymorphic) polymorphic genotypes.

Table 1

Genotypes of *Aurora-A* and *p16* and their association with risk for early diagnosis of pancreatic cancer

Genotype	*n* (%), *N* = 148	Median age at diagnosis (y)	*P**	HR (95% CI)
Aurora-A (T→A)				
TT	101 (68.2)	62.4	0.015	1.00 (reference)
TA/AA	47 (31.8)	59.6		1.55 (1.09–2.20)
p16 C540G (C→G)				
CC	107 (72.3)	60.4	0.252	1.00 (reference)
CG/GG	41 (27.7)	61.4		0.80 (0.56–1.17)
p16 C580T (C→T)				
CC	120 (81.1)	62.1	0.050	1.00 (reference)
CT/TT	28 (18.9)	58.9		1.52 (1.00–2.30)

* Log-rank test for homogeneity between genotypes.

Table 2

Joint effects of polymorphisms of *Aurora-A* and *p16* on risk for diagnosis of pancreatic cancer at an early age

Genotypes *Aurora-A* (T→A)	***p16* C580T** (C→T)	***n* (%), *N* = 148**	**Median age at diagnosis (y)**	**HR (95% CI)**
TT	CC	83 (56.1)	63.4	1.00 (reference)
TT	CT/TT	18 (12.2)	59.1	1.29 (0.77–2.15)
TA/AA	CC	37 (25.0)	60.5	1.41 (0.95–2.10)
TA/AA	CT/TT	10 (6.7)	50.8	3.88 (1.94–7.76)*

*$P < 0.05$, departure from additive model.