CDKN2A Common Variants and Their Association with Melanoma Risk: A Population-Based Study

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Abstract

The population frequencies of the CDKN2A variants remain undetermined. In Poland there are three common variants of CDKN2A: an alanine to threonine substitution (A148T), Nt500c>g and Nt540c>t, which have been detected in other populations. To establish if they are associated with an increased malignant melanoma (MM) risk we did an association study based on genotyping 471 patients with MM and 1,210 random control subjects from the same Polish population. We found a significantly increased frequency of the A148T variant among patients with MM (7.0%) in comparison with the general population (2.9%). The incidence of the A148T variant remained greater in both unselected and familial melanoma subgroups. A statistically significant positive association was seen for unselected MM (odds ratio, 2.529; P = 0.0003), especially in patients diagnosed under 50 years of age (odds ratio, 3.4; P = 0.0002). The A148T carrier population (homozygous G/A alleles) was more likely to have a relative with malignancy compared with the noncarrier population (57% versus 36%, respectively; P = 0.03). Further examination of the CDKN2A1 promoter sequence done in 20 melanoma patients with the A148T change (heterozygous G/A alleles) and 20 patients with MM without this alteration identified it was in linkage disequilibrium with a polymorphism in the promoter region at position P-493. We found no statistically significant overrepresentation of the Nt500c>g and the Nt540c>t polymorphisms in the Polish melanoma population. In conclusion, the A148T variant of the CDKN2A gene seems to be associated with an increased risk of development of MM. Additional studies are required to confirm whether this particular change is associated with increased risk of other nonmelanoma malignancies. (Cancer Res 2005; 65(3): 835-9)

Introduction

Cutaneous and ocular malignant melanomas (MM) represent one of the most aggressive neoplasms and their frequency is increasing rapidly (1, 2). The genetic basis of MM is complex and seems to involve multiple genes. CDKN2A (OMIM 60160) was the first to be associated with MM risk and is regarded as the major MM susceptibility gene (3). Its protein product p16 is a cyclin-dependent kinase inhibitor that suppresses cell proliferation (4).

Germ line mutations of the CDKN2A have been detected with varying frequencies (6-48%) in small samples of French (5), U.S. (6), Australian (7), Dutch (8), Italian (9), Swedish (10), English (11), and Polish melanoma-prone families (12, 13). Thus far, there have been 77 causative CDKN2A variants listed in the international melanoma mutation database, eMelanoBase. The frequency of these CDKN2A variants in the reported populations remains undetermined; however, because they result in an altered protein product they are considered to account for rare familial forms of the disease.

Two common polymorphisms in the 3’ untranslated region of CDKN2A have also been described as being associated with a modulation of risk (14) or disease progression (15). However, one study indicates that there is no overrepresentation of the Nt500c>g and the Nt540c>t variants in the melanoma population (16).

In Poland, apart from the two polymorphisms in the 3’ untranslated region, there is one more common variant of CDKN2A—an alanine to threonine substitution at codon 148 (A148T)—which has been estimated to be present in approximately 3% to 3.5% of the population (12, 13). Functional studies suggest this variant is a polymorphism, which seems to have no major effect on p16 function (17, 18). Previous studies have shown that the A148T polymorphism is in linkage disequilibrium with a promoter polymorphism P-493, which has been shown to affect gene expression (19, 20). Nevertheless, the A148T change has been found to be overrepresented in melanoma kindreds (3%) in comparison to the general population (1.8%; ref. 16).

To establish if the A148T, Nt500c>g, and the Nt540c>t variants are associated with increased melanoma risk we did an association study based on genotyping 471 patients with MM and 1,210 random control subjects from the same Polish population.

Patients and Methods

Patients. The unselected case group consisted of 471 patients with MM (264 females, 207 males; mean age at onset, 54.5 years; age range, 20-85 years), comprising 301 unselected patients with MM (mean age, 54.7 years; range, 26-78 years) diagnosed in northwestern Poland between 2000 and 2003 (Szczecin, Gorzów Wlkp, Zielona Góra), 80 unselected consecutive MM cases (mean age, 53.2 years; range, 29-74 years) diagnosed between 2000 and 2003 in northeastern Poland (Białystok), and 90 unselected consecutive MM cases (mean age, 54.1; age, 34-79) diagnosed between 2002 and 2003 in southwest Poland (Opole).

All MM cases were taken from cancer registries in the five cities mentioned above. Participation rates exceeded 75% for Białystok and Opole. In northwestern Poland (301 unselected
cases), participation rate exceeded 75%; in Szczecin (214 cases regarded as unselected consecutive MM), the rate exceeded 50% but not 75% in Gorzów Wlkp (47 cases regarded as unselected but nonconsecutive) and Zielona Góra (40 cases regarded as unselected but nonconsecutive).

Among 471 unselected cases, 56 patients (11.9%) had a first- or second-degree relative affected with MM of which 14 patients (3.0%) had a first-degree relative affected with MM (familial melanoma cases).

The control population consisted of 500 consecutive newborns from the clinical hospitals of Szczecin and 710 controls selected at random from the computerized patient lists of five family practices in Szczecin, Białystok, and Opole (363 females; mean age, 52.3 years; range, 21-77 years) and 347 males (mean age, 54.7 years; range, 19-81 years). To ensure comparability with the control groups, the allele frequencies of the three alleles under study was computed separately for the adult and neonatal control groups.

Materials. DNA samples were obtained from peripheral blood of individuals or from umbilical chord blood of newborns.

The A148T variant was analyzed by RFLP PCR, using np16ex2f (AGGGTGTTAGACACTCTG) and np16ex2r (TTITGGAAGCTCT-CAGGGTAC) primers. PCR products were digested with the SacII enzyme and separated in 2% to 3% agarose gels.

The Nt500c>g and the Nt540c>t variants were analyzed by RFLP PCR, using np16ex3f (TGAAGCCATTGCGAGAACTT) and np16ex3r (TCTACGTTAAAAGGCAGGAC) primers. PCR products were digested with the AvaI and HaeIII enzymes and separated in 2% to 3% agarose gels.

In cases positive in RFLP PCR, DNA samples were sequenced to confirm the presence of the mutation. Sequencing was done with BigDye Terminator Ready Reaction kit and analyzed in ABI PRISM 377 DNA sequencer (Applied Biosystems).

Additional analysis of the P-493 polymorphism was based on genomic sequencing of CDKN2A promoter sequence done using primers P96F (AAAGCAGGGGGCACTCATATTC) and P968R (TCCGAGCACTTAGCGAATGT).

Statistics. To evaluate whether identified CDKN2A alterations were associated with MM we compared the frequency of occurrence of detected variant in our patients with the control group from the general population, using two-sided Fisher’s exact test. The patient cohort was also further analyzed by dividing the MM group into those persons <50 years of age and comparing the frequency of the variants observed in this group to those >50 years of age. The age determinant of 50 years was chosen because those cases <50 years of age were more likely to be due to genetic influences compared with those patients >50 years of age whose disease was more likely to be a result of environmental exposure.

Table 1. Frequencies of CDKN2A variant alleles in controls and cases

<table>
<thead>
<tr>
<th>Allele</th>
<th>Frequency</th>
<th>OR (95% CI)</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>A148T</td>
<td>Newborns</td>
<td>Adults</td>
<td>Total controls</td>
</tr>
<tr>
<td></td>
<td>(n = 500)</td>
<td>(n = 710)</td>
<td>(n = 1,210)</td>
</tr>
<tr>
<td>A/A</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>G/A</td>
<td>14 (2.8%)</td>
<td>21 (2.95%)</td>
<td>33 (7%)</td>
</tr>
<tr>
<td>G/G</td>
<td>486 (97.2%)</td>
<td>689 (97.1%)</td>
<td>689 (97.2%)</td>
</tr>
<tr>
<td>Allele A frequency</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Nt500c&gt;g</td>
<td>Newborns</td>
<td>Adults</td>
<td>Total controls</td>
</tr>
<tr>
<td></td>
<td>(n = 500)</td>
<td>(n = 710)</td>
<td>(n = 1,210)</td>
</tr>
<tr>
<td>A/A</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>G/G</td>
<td>8 (1.2%)</td>
<td>9 (1.3%)</td>
<td>15 (1.2%)</td>
</tr>
<tr>
<td>G/A</td>
<td>88 (17.6%)</td>
<td>143 (20.1%)</td>
<td>229 (18.9%)</td>
</tr>
<tr>
<td>G/C</td>
<td>406 (81.2%)</td>
<td>558 (78.6%)</td>
<td>966 (79.8%)</td>
</tr>
<tr>
<td>Allele A frequency</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
</tbody>
</table>

*P > 0.5, non significant.

1P = 0.0003.

2P = 0.0002.

3P = 0.0315.
Possible deviation of the genotype frequencies from those expected under Hardy-Weinberg equilibrium was assessed by the \( \chi^2 \) probability test (21).

**Results**

The frequency of the A148T change was assessed in the two control populations (newborns versus adults) and the frequencies were 2.8% and 2.95% (Table 1). These were not statistically different \((P = 1.0)\). Further verification of the similarities of the two control populations were undertaken by examining a common CHEK2 polymorphism. There was no statistical difference in the CHEK2 allele frequencies in the adult controls recruited from the Szczecin metropolitan region compared with other Polish cities (data not shown). It was then considered justified to use the combined control population frequency of 2.89% for statistical analysis.

There were no large differences in the frequencies of the Nt500c>t alleles and in the frequencies of the Nt540c>t alleles in the two control populations (Table 1). Because these were not statistically significant (data not shown), combined control population frequencies were used for statistical analyses of both of these CDKN2A changes.

There was no evidence that the genotype frequencies of the three CDKN2A variants deviated from those expected under Hardy-Weinberg for the control group or any of the melanoma groups \((P > 0.4)\).

In the melanoma population under study the frequency of the A148T variant was significantly greater than that observed in the control population (see Table 1) and it was associated with a significant increase in the odds ratio (OR) of developing melanoma.

To further determine the importance of the A148T change, its frequency was assessed in patients who were <50 years of age and compared with those >50 years. The results reveal that the frequency is greater in the <50-year-old group compared with the >50-year-old group (Table 1). The OR was consequently greater in the younger age group (OR, 3.5; \( P = 0.0007 \)) compared with the older group (OR, 2.1; \( P = 0.0351 \)). There was no statistical difference between the frequency and OR of the polymorphism between both groups (OR, 1.6; \( P = 0.265 \)).

The clinical features of patients harboring the A148T change were compared with patients who did not. The mean age of A148T carriers was 53 years and the mean age of noncarriers was 55 years; this was not statistically significant \((P = 0.632)\).

Among the cancers seen in the first-degree relatives of the mutation-positive melanoma patients were stomach (2 cases), larynx (2), pancreas (2), lung (3), breast (1), female genital (3), bladder (3), and skin (2) cancers and single cases of colon, kidney, and liver cancer.

To further define the association of the A148T change and its link to malignancy we examined the occurrence of cancer of any type in first-degree relatives of carriers compared with noncarriers. Familial melanoma cases were excluded from this evaluation. This analysis revealed that the carrier population was more likely to have a relative with malignancy compared with the noncarrier population (57% versus 36%, respectively; \( P = 0.03 \)).

Because there is doubt about the functional consequence of the A148T change, but it is in linkage disequilibrium with a promoter polymorphism that affects gene expression levels, 20 A148T heterozygous carriers and 20 A148T noncarriers were studied to

<table>
<thead>
<tr>
<th>Allele T frequency</th>
<th>OR (95% CI) Frequency</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.6%</td>
<td>4 (0.8%) T/T</td>
<td>1.102* (0.2836-4.280)</td>
</tr>
<tr>
<td></td>
<td>62 (12.4%) C/T</td>
<td>1.155* (0.8424-1.584)</td>
</tr>
<tr>
<td></td>
<td>434 (86.8%) C/C</td>
<td>0.866* (0.6357-1.181)</td>
</tr>
<tr>
<td>7.4%</td>
<td>3 (0.6%) T/T</td>
<td>1.142* (0.8526-1.528)</td>
</tr>
<tr>
<td></td>
<td>64 (13.5%) C/T</td>
<td>2.022* (0.4164-9.816)</td>
</tr>
<tr>
<td></td>
<td>404 (85.7%) C/C</td>
<td>1.021* (0.6267-1.665)</td>
</tr>
<tr>
<td>6.6%</td>
<td>2 (1.2%) T/T</td>
<td>1.014* (0.6708-1.584)</td>
</tr>
<tr>
<td></td>
<td>21 (12.2%) C/T</td>
<td>0.931* (0.5811-1.491)</td>
</tr>
<tr>
<td></td>
<td>149 (86.6%) C/C</td>
<td>1.114* (0.1719-1.727)</td>
</tr>
<tr>
<td>7.3%</td>
<td>1 (0.3%) T/T</td>
<td>0.857* (0.0706-4.708)</td>
</tr>
<tr>
<td></td>
<td>43 (14.4%) C/T</td>
<td>1.234* (0.8549-1.780)</td>
</tr>
<tr>
<td></td>
<td>255 (85.3%) C/C</td>
<td>0.833* (0.5795-1.196)</td>
</tr>
<tr>
<td>7.5%</td>
<td>Allele T frequency</td>
<td>1.157* (0.8205-1.632)</td>
</tr>
</tbody>
</table>

Table 1. Frequencies of CDKN2A variant alleles in controls and cases (Cont’d)
Cancer Res 2005; 65: (3). February 1, 2005 838 www.aacrjournals.org

3.
Hashemi J, Platz A, Ueno T, Stierner U, Ringborg U,

References


Discussion

In a previous study we identified the A148T variant (Nt442A) in a group of familial melanoma patients and melanoma patients with familial aggregations of breast cancer (12).

This is the first case-control study that shows an association between 148T alteration and increased risk of melanoma. The A148T variant is broadly cited as a common polymorphism on the basis of two observations: functional studies indicating that this amino acid substitution does not affect the ability of the p16 to precipitate with CDK4/CDK6 proteins (17, 18) and it does not segregate with melanoma in melanoma-prone families (22, 23). Some authors have reported an increased incidence of the A148T variant in small cohorts of melanoma-prone families (6, 11). It has been found to be present in 4% of the Utah population (24), 3% of parents of the Centre d’Etude Polymorphisme Humain (22), and 1.8% of the Queensland population (16). In all instances, these studies have focused on relatively small numbers of patients; even in the larger study done by Aitken et al. (16) the number of control subjects did not exceed 200. In the current study, the frequency of the A148T change in 1,210 control samples was found to be 3%.

Population stratification might explain significant overrepresentation of the A148T allele in the Polish population. In a recent study Acton et al. found that the frequencies of Celtic ancestry and associated marker HLA-DRB1*04 were statistically greater in the melanoma cases (25). It seems that there are no major population substructures within Poland. First, most of the cases used in the present study were recruited from the northwest region, which is populated by ethnic Poles who migrated to the region from throughout Poland. Second, there were no significant differences in the CDKN2A allele frequencies in the 3 examined regions of Poland. Finally, the similar frequency of the three polymorphisms identified in the newborn and adult populations indicates that the polymorphisms are in genetic equilibrium. These findings and those published previously by our group (26, 27) suggest that the Polish population is homogeneous. On the other hand, we observed a trend of higher G allele frequency, although statistically nonsignificant, for Nt500 variant in late-onset melanoma cases (P = 0.0664). This could point at small numbers of cases or possible population stratification. Thus it is necessary to determine the distribution of genotypes at a number of other unrelated loci throughout Poland. To evaluate the hypothesis that A148T aggregates with country of ancestry, other Slavic populations should be examined.

Because the A148T change has not been shown to alter protein function yet seems to be associated with disease it is most likely that this polymorphism is in linkage disequilibrium with another alteration that does affect protein function. Indeed, there are several reports indicating that the A148T polymorphisms is in linkage disequilibrium with a change in the promoter region at position P-493 (19, 20) and the Nt540T in the 5’ untranslated region (16). It is beyond the scope of this study, but it would be interesting to evaluate different common polymorphisms, such as the Nt191 in the 5’ untranslated region (28). Further examination of the CDKN2A promoter sequence done in 20 melanoma patients with A148T change and 20 patients with MM without this alteration supports the findings of others that linkage disequilibrium exists between the A148T polymorphism and the P-493 polymorphism. These results indicate that the Polish population under study is not significantly different to other European populations with respect to the A148T change and the P-493 polymorphism.

The results of our study indicate that in the Polish population the A148T variant is associated with an increased melanoma risk especially for patients diagnosed under 50 years of age (OR, 3.5). The A148T can be added to the list of DNA variants, which are believed to predispose to MM. Others include the melanocortin-1 receptor (MCIR) gene variants, which were the first reported to be influencing melanoma risk independently of fair skin and red hair (29). Polymorphisms in the nucleotide excision repair gene XPD have also been reported to be associated with an increased risk of melanoma (30, 31).

The frequencies of the two 3’ untranslated region polymorphisms recorded in the Polish control population are similar to those reported in the Nordic healthy control population (14). However, we found lower incidences of these polymorphisms among melanoma patients in comparison with Nordic patients affected with this disease. The results of our statistical analyses are consistent with data presented by Aitken et al. (16) in that we did not find any significant increase of the two polymorphisms in our melanoma population and that for each polymorphism the rare allele frequency was higher in melanoma cases than controls.

In conclusion, the A148T variant of the CDKN2A gene seems to be associated with an increased risk of developing MM. Nevertheless this does not imply that the A148T polymorphism is associated with increased disease risk alone but rather suggests there are interactive modifiers that when taken together result in malignancy. Additional studies are required to determine whether this particular change can be associated with the increased risk of malignancies at different sites of origin as suggested by the increased frequency of cancer in first-degree relatives of A148T carriers.

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