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The *CDKN2a* common variants: 148 Ala/Thr and 500 C/G in 3′ UTR, and their association with clinical course of melanoma

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Changes in *CDKN2a* gene are known to be linked with sporadic melanoma and hereditary predisposition to this cancer. In the Polish population mutations in the coding region of the *CDKN2a* gene are rather rare, therefore the attention has been focused on polymorphisms and alterations in uncoding regions such as 3′ UTR. The aim of this study was to analyze two common polymorphisms, Ala148Thr and 500 C/G, and correlate them with the clinical course of melanoma. DNA from 285 patients was analyzed and found polymorphisms were correlated with the clinical parameters employing statistical methods. The obtained results allow us to conclude: (i) survival times of 500 C/G carriers *vs*. cumulating proportion surviving was not statistically significant; (ii) *CDKN2a* polymorphism 500 C/G correlated with Ala148Thr; (iii) no correlation was observed between the 500 C/G polymorphism and age of diagnosis, localization of primary melanoma and survival time; (iv) we did not find correlation between 500 C/G and type of cancer in the family; (v) changes in the *CDKN2a* gene were not found in patients with second cancer.

Keywords: melanoma, *CDKN2a* polymorphisms, statistical analysis, 3′ UTR

INTRODUCTION

The etiology of cutaneous melanoma is heterogeneous and involves genetic predisposition and environmental factors. Melanoma is a genetically complex disease involving a large number of genes. *CDKN2a* (OMIM*600160) was identified as the first high-penetrance melanoma susceptibility gene linked with family history of melanoma, young age of onset or multiple primary tumors. The *CDKN2a-ARF* locus is located on chromosome 9p21 and encodes two distinct proteins involved in cell cycle regulation. p16, one of the protein products of the *CDKN2a-ARF* gene, is a small cyclin-dependent kinase inhibitor. Germline mutations in the *CDKN2a* appear in varying frequencies from 5 to 50% in different populations and are more frequent in families with more than three cases of melanoma. On the other hand, mutations in the *CDKN2a* gene are rather rare, and the number of carriers depends on the geographic area, closely connected with such risk factor as level of UV radiation. Such relationship was confirmed by frequently detected mutations in codon 61 of a *RAS* gene in the *CDKN2a* variant carriers (Eskandarpour *et al.,* 2003). In the Polish population, mutations in the *CDKN2a* gene are very rare (Lamperska *et al.,* 2002; Dębniak *et al.,* 2005). Accordingly, more attention has been focused on untranslated regions of the gene (UTRs) and their polymorphisms. Studies carried out on British and Australian populations

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Abbreviations: CDKN2a, cyclin-dependent kinase inhibitor 2a; FAMMM, familial atypical mol-malignant melanoma syndrome; FAMMM-PC, familial atypical; mol, malignant melanoma-pancreatic carcinoma syndrome; MPM, multiple primary melanomas; PCR-SSCP, PCR single strand conformational polymorphism.

have demonstrated genetic changes in the untranslated regions, some related to melanoma incidence. Examples include G/T substitution in position –34 in 5′ UTR generating a new AUG start codon. In British and Australian families two variants of intron sequences IVS1 +1104 C/A and IVS1 —1104 C/G which both predispose to melanoma were found (Harland *et al.*, 2005). The *CDKN2a* gene also carries polymorphisms. The most common sites 500 C/G and 540 C/T are located in 3′ UTR (Kumar *et al.*, 1998; 2001) and 148 Ala/Thr in exon 2. Functional analysis indicated that this amino acid change did not affect p16 ability to inhibit CDK4 enzymatic activity (Ranade *et al.*, 1995; Reymond & Brent, 1995). It is very difficult to directly asses the effect of changes in 3′ UTR on p16 mRNA metabolism and its connection with melanoma development and progression. Indirect functional studies such as assessment of correlation of p16 polymorphisms and clinical features of the disease may provide some insight into mechanisms involved. Accordingly, the aim of the present study was to analyze two common polymorphisms in the *CDKN2a* gene and correlate them with the clinical course of the disease.

MATERIALS AND METHODS

Blood samples were collected from 425 melanoma patients but only in 285 individuals clinical features were assessed. They included: age of first diagnosis, tumor localization, survival time, age of death, and familial history. Clinical parameters were correlated with the diagnosed polymorphisms employing statistical methods.

DNA isolation. DNA was isolated from whole blood samples or from lymphocytes stored in liquid nitrogen using Wizard genomic extraction kit (Promega, WI, USA) according to the manufacturer's instruction.

PCR-SSCP analysis. The following sets of primers were used: for 148 Ala/Thr 5′-TGGACGTGCGCGCGATGC and 5′-GGAAGCTCTCAGGGTACAAATTC, for 3′ UTR polymorphisms 5′-GACATCCCCGATTGAAAGAACC and 5′-ATTTACGGTAGTGGGGGAAGGC. Primers were labeled on the 5′ end with [γ-^{32}P]ATP (3000 Ci/mmol, Amersham). The reaction volume for PCR was 5 μl and included 1× PCR buffer, 1.5 mM $MgCl_2$, 0.8 mM dNTPs, 1 μM of appropriate primers, 0.125 U of *Taq* DNA polymerase and 50 ng of genomic DNA. The amplification program consisted of three steps PCR profile with annealing temperature 55°C. After PCR the samples were mixed with 1:5 volume of formamide dye, heated prior to loading into a gel (6% polyacrylamide gel with 10% glycerol). Electrophoresis was carried out at room temperature at 15 W. Then the gels were transferred onto Whatman 3MM paper, dried and autoradiographed.

Sequencing analysis. Bands showing atypical mobility shift were cut out from the gel, eluted into water and amplified with the same pair of primers. Products of PCR reaction were purified and served as sequencing template. Sequencing reactions were performed using the fmol sequencing kit (Promega).

Statistical analyses. Statistical analysis was performed using univariate logistic regression. We used Shapiro–Wilk and Lilliefors test and then Student' *t*-test, χ^2 test with Yates' modifications and Fisher's exact test. The Mann–Whitney U-test served as a nonparametric method. The statistical significance of analyzed factors depending on patient survival or death was estimated. Survival probability was estimated by the Kaplan–Meier method. Data showing statistical significance was correlated with survival time. The statistical significance of prognostic factors depending on survival time was assessed by the Cox nonparametric proportional hazard regression model. A P value < 0.05 was considered as statistically significant. The statistical analysis was performed using Statistica for Windows release 6.0.

RESULTS

In a group of 285 patients with melanoma some had secondary tumors: one patient had multiple primary melanomas (MPM), five had breast cancer, one had colon cancer, one patient had uterus cancer, one had retinoblastoma and finally one had two different cancers: breast and thyroid. Melanoma was localized in 127 cases on the trunk, in 112 on a limb, in 27 cases on the head, in 2 in an eye, in 2 under a nail, one in rectum and in 14 cases the primary localization was unknown. One-hundred and thirty-two patients died during observation, while most of them (104 cases) within 5 years following melanoma diagnosis. The average age of first diagnosis in the examined group was 48.13 years (range: 19 to 80, S.D. 13.58). The group included 155 women (54.39%) and 130 men (45.61%).

Survival of over eight years was observed in seven cases. In eight cases the exact date of death was not known. The correlation between clinical parameters and survival is present in Table 1. The median survival time according to Kaplan–Meier was 90.87 months, lower quartile (25th percentile) was 42.90 months. The curve of survival is showed in Fig. 1.

Fifty-nine patients reported occurrence of cancer in the family. Melanoma was reported by 12 families (20.33%), however, two melanomas in a family were recognized only in two cases. Among other types of cancers there were: uterus cancer in 8

Table 1. Correlation between survival time and clinical parameters

Variables		0 (death)	1 (survival)	*P* (value)
Gender	Female	68 51.51%	87 56.86%	NS
	Male	64 48.49%	66 43.14%	
Age of first diagnosis		Mean 48.14; st. dev. 14.14, range 20–79	Mean 48.11; st. dev. 13.12, range 19–80	NS
Tumor localization				
trunk, shoulder girdle or neck		60	67	NS
limbs		53	59	NS
head		8	19	NS
subungual		2	0	NS
anus		1	0	NS
eye		2	0	NS
unknown		6	8	NS

NS, non significant

families (13.6%), lung cancer in 17 families (28.8%), stomach in 9 (15.2%), colon in 6 (10%), larynx in 6 (10%), liver in 4 (6.8%), pancreas in 3 (5%), brain in 3 (5%), and single incidence of other types of cancer in 10 families, finally, two patients reported FAMMM (OMIM#155600). Although 20.7% of patients had cancer incidence in family history, only two cases could be qualified as syndromes predisposing to melanoma: one of familial melanoma malignum and the second FAMMM-PC (OMIM#606719). The correlation between cancer family history and survival time is presented in Table 2.

PCR-SSCP analysis demonstrated the 500 C/G variant in 62 cases and 148 Ala/Thr polymorphism in seven cases. Both changes together were present in five cases, and the correlation between the variants 500 C/G and 148 Ala/Thr was statistically significant (P=0.0066). Patients with multiple cancers including melanoma did not show polymorphisms in the *CDKN2a* gene. All patients with coexisting melanoma and breast cancer were investigated for the presence of mutations in the *BRCA1* gene, characteristic for the Polish population (Górski *et al.*, 2000). No changes in *BRCA1* were found. DNA from patients reporting breast cancer in the family was also analyzed for mutations in the *BRCA1* gene, but such changes were not found. Polymorphism 500 C/G was found in nine patients having cancers in the family, but no correlation with the type of cancer was established. 148 Ala/Thr was present in only one patient with a family cancer history, together with the 500 C/G variant. Since 148 Ala/Thr was recognized mostly in DNA from patients with no cancer history in the family, correlation analysis between the variant and the type of cancer in the family was not performed. At the time of analysis, 153 patients were alive while 132 had died. Polymorphism 500 C/G was found in 26 living persons and 37 dead. The correlation between the percentage of dead patients and the 500 C/G polymorphism was found to be statistically significant (P=0.0252) (Table 3). No correlation was observed for 148 Ala/Thr (P=0.5608) alone nor for the

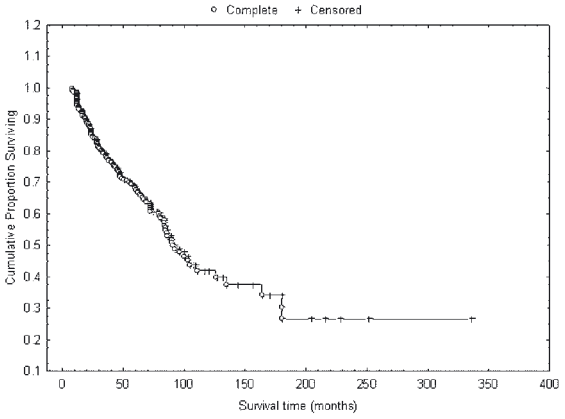

Figure 1. Survival time of melanoma patients *vs.* cumulative proportion surviving according to Kaplan–Meier method.

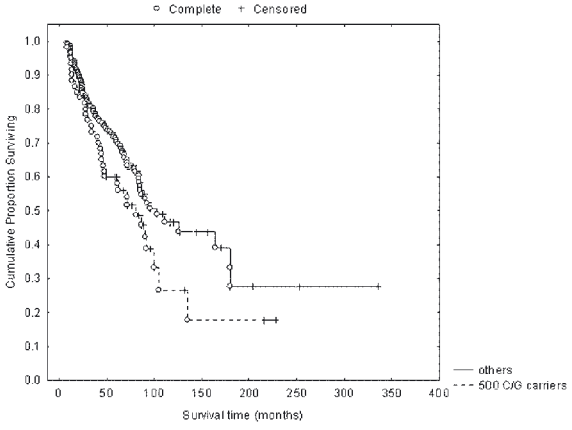

Figure 2. Survival time of 500 C/G carriers *vs.* cumulative proportion surviving according to Kaplan–Meier method.
Log-Rank Test; P = 0.0531.

Table 2. Correlation between cancer family history and survival time

Variables	0 (death)	1 (survival)	*P* (value)
One case of melanoma malignum	1	9	Chi^2 *P*= 0.0191[a]
	131	144	Fisher: *P*=0.0176[a]
More than one case of melanoma malignum	0	2	NS Chi^2 *P*= 0.1874
	132	151	Fisher: *P*=0.2873
FAMMM	0	2	NS Chi^2 *P*= 0.1874
	132	151	Fisher: *P*=0.2873
One case of familial breast cancer history	5	7	NS Chi^2 *P*= 0.7414
	127	146	Fisher: *P*=0.4892
More than one case of breast cancer history	0	1	NS Chi^2 *P*= 0.3521
	132	152	Fisher: *P*=0.5368
Carcinoma uteri	2	6	NS Chi^2 *P*= 0.2200
	130	147	Fisher: *P*=0.1947
One case of lung cancer history	7	7	NS Chi^2 *P*= 0.7768
	125	146	Fisher: *P*=0.4941
More than one case of lung cancer history	3	0	NS Chi^2 *P*= 0.0609
	129	153	Fisher: *P*=0.0981
Larynx cancer	4	2	NS Chi^2 *P*= 0.3123
	128	151	Fisher: *P*=0.2754
Gastric cancer	4	5	NS Chi^2 *P*= 0.9089
	128	148	Fisher: *P*=0.5913
Colon cancer	1	1	NS Chi^2 *P*= 0.1410
	131	152	Fisher: *P*=0.1452
Pancreatic cancer	2	2	NS Chi^2 *P*= 0.4773
	130	151	Fisher: *P*=0.4446
Hepatic cancer	2	2	NS Chi^2 *P*= 0.8817
	130	151	Fisher: *P*=0.6314
Brain tumors	1	2	NS Chi^2 *P*= 0.6503
	131	151	Fisher: *P*=0.5554
Other cancers	4	6	NS Chi^2 *P*= 0.6835
	128	147	Fisher: *P*=0.4695

[a]Statistically significant; NS, non Significant

occurrence of both changes together (*P*=0.0509). The overall survival did not correlate with the presence of the 500 C/G or 148 Ala/Thr, polymorphisms, although in Log-Rank Test the survival times of 500 C/G carriers demonstrated a trend of significance (*P*=0.0531). Graphs of survival times of 500 C/G and

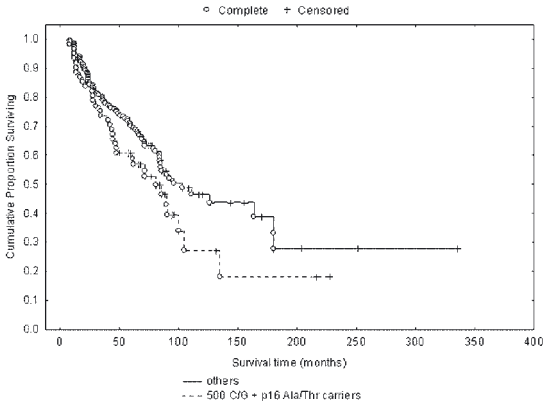

Figure 3. Survival time of 500 C/G + 148 Ala/Thr carriers *vs.* cumulative proportion surviving according to Kaplan–Meier method.
Log-Rank Test; *P*= 0.0707.

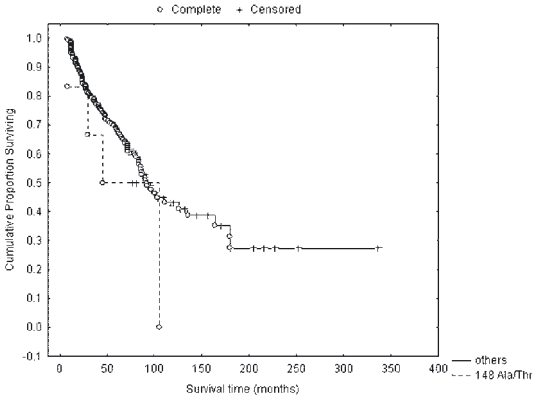

Figure 4. Survival time of 148 Ala/Thr carriers *vs.* cumulative proportion surviving according to Kaplan–Meier method.
Log-Rank Test; *P*= 0.3105.

Table 3. *CDKN2a* polymorphisms and number of deaths and survivals

Variables	0 (death)	1 (survival)	*P* (value)
500 G/C	37	26	Chi^2 P = 0.0252 [a]
	95	127	Fisher: P = 0.0181[a]
500 G/C and 148 Ala/Thr	37	28	Chi^2 P= 0.0509 [a]
	95	125	Fisher: P= 0.0352 [a]

[a]Statistically significant.

500 C/G plus 148 Ala/Thr, and of 148 Ala/Thr *vs*. cumulative proportion surviving according to the Kaplan–Meier method are presented in Figs. 2, 3 and 4.

The median age of diagnosis of all the patients was 47 years, while for the carriers of the 500 C/G variant it was 50. No correlation between the age of diagnosis and the presence of the 500 C/G variant was found (P=0.5984)**.** No correlation was found for carriers of the 148 Ala/Thr polymorphism and the age of melanoma diagnosis (P=0.2358).

DISCUSSION

One of the most significant risk factors is the family history of melanoma, reported by about 10% of melanoma patients. In our studies melanoma appeared only in 12 families, but only two reported up to 2 cases in interview. We did not observe a correlation between the occurrence of polymorphisms in *CDKN2a* and the familial history of melanoma, most likely due to the limited number of patients in the investigated group. For the same reason no correlation was observed for patients with FAMMM and MPM (only one case). Then we correlated recognized polymorphisms with spectrum of cancers in familial history. It is known that the 148 Ala/Thr variant predisposes to melanoma and associates with breast cancer in the Polish population (Dębniak *et al.*, 2005). Moreover, an epidemiologic study has provided evidence of a link between melanoma and breast cancer developing in the same person (Goggins *et al.*, 2004). Carriers of mutations in the *BRCA2* gene exhibit increased risk of melanoma while carriers of mutations in *CDKN2a* have increased risk of breast cancer. We also observed a coincidence between the presence of melanoma and breast cancer in the same patient, but no mutations in *CDKN2a* and *BRCA1* were found. In a population-based study it has been shown that 148 Ala/Thr heterozygous carriers were more likely to have a first-degree relative with a cancer of any type compared to non-carriers: 57% *versus* 36%, respectively (Dębniak *et al.*, 2005). We did not observe a correlation between 148 Ala/Thr and the occurrence of cancers in the family. The above variant was found only in one patient with a familial history of cancer. We did not find a correlation between the 500 C/G allele and the type of cancer in a patients' family.

Finally, we looked for correlations between the investigated alleles and the clinical course of melanoma. Such analysis was carried out for polymorphic variants found in DNA isolated from melanoma tissue. In tumors showing loss of heterozygosity the polymorphic allele was usually retained. Polymorphisms in 3′ UTR are regarded to be connected with a significantly shorter progression time from primary to metastatic disease (P=0.0071) (Sauroja *et al.*, 2000). We found a correlation between the percentage of patients dying and the 500 C/G variant, however, when the survival time of patients with the C/G variant *vs*. cumulative proportion surviving were analyzed, the P value was not statistically significant. We did not find a correlation between the presence of the 500 C/G polymorphism and the age of diagnosis or localization of primary melanoma.

The phenomenon that the 500 C/G variant appearing more frequently in DNA of melanoma patients is still unknown. The association of *CDKN2a* polymorphisms with melanoma risk is not strong (Aitken *et al.*, 1999) but the frequency of the 500 C/G polymorphism showed to increase with an increasing family risk group. In our investigation we did not answer the question how the 500 C/G variant is correlated with melanoma, but the obtained results allow us to conclude that: (i) survival times of 500 C/G carriers *vs*. cumulating proportion surviving was not statistically significant; (ii) the 500 C/G polymorphism correlated with 148 Ala/Thr; (iii) no correlation was observed between the presence of the 500 C/G polymorphism and age of diagnosis, localization of primary melanoma and a survival time; (iv) we did not find a correlation between 500 C/G and type of cancer in the family; (v) no changes in a *CDKN2a* gene were found in patients with a second cancer.

Acknowledgements

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