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Some Molecular and Clinical Aspects of Genetic Predisposition to Malignant Melanoma and Tumours of Various Site of Origin

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Key words: *CDKN2A*, *ARF*, *XPD*, *MC1R*, melanoma, breast cancer, family history, age at diagnosis, cancer risk, mutation analysis, Poland

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Abstract

Based on epidemiological data we can assume that at least some malignant melanoma (MM) and breast cancer cases can be caused by the same genetic factors. *CDKN2A*, which encodes the p16 protein, a cyclin-dependent kinase inhibitor suppressing cell proliferation, is regarded as a major melanoma susceptibility gene and the literature has also implicated this gene in predisposition to breast cancer. Genes also known to predispose to MM include *XPD* and *MC1R*. We studied *CDKN2A/ARF*, *XPD* and *MC1R* for their associations with melanoma and breast cancer risk in Polish patients and controls. We found that *CDKN2A* and *ARF* do not contribute significantly to either familial melanoma or malignant melanoma within the context of a cancer familial aggregation of disease with breast cancer. However, the common variant of the *CDKN2A* gene A148T, previously regarded as non-pathogenic, may predispose to malignant melanoma, early-onset breast cancer and lung cancer. Compound carriers of common *XPD* variants may be at slightly increased risk of breast cancer or late--onset malignant melanoma. Common recurrent variants of the *MC1R* gene (V60L, R151C, R163Q and R160W) may predispose to malignant melanoma. In general, the establishment of surveillance protocols proposed as an option for carriers of common alterations in *CDKN2A*, *XPD* or *MC1R* variants requires additional studies. It is possible that missense variants of genes for which truncating mutations are clearly pathogenic may also be deleterious, but with reduced penetrance. This may be overlooked unless large numbers of patients and controls are studied. A registry that includes 2000 consecutive breast cancer cases, 3500 early onset breast cancer patients, 500 unselected malignant melanoma and over 700 colorectal cancer patients has been established in the International Hereditary Cancer Centre and can contribute to these types of large association studies.

Introduction

Based on epidemiological data we can assume that at least some malignant melanoma (MM) and breast cancer cases can be caused by the same genetic factors. *CDKN2A*, which encodes the p16 protein, a cyclin-dependent kinase inhibitor suppressing cell proliferation, is regarded as a major melanoma susceptibility gene and the literature has also implicated this gene in predisposition to breast cancer. Genes also known to predispose to MM include *XPD* and *MC1R*. This paper reviews a range of studies which have been performed in Polish populations with the following objectives:

1. Assessment of the risk of malignancies of various sites of origin in relatives of malignant melanoma patients from families with strong familial cancer aggregation.
2. Assessment of germline mutation and large deletion analysis of the *CDKN2A/ARF* genes in families with multiple melanomas and in families with an aggregation of malignant melanoma and breast cancer.
3. Determination of the association of *CDKN2A* common variants with melanoma risk.
4. Assessment of breast cancer risk among carriers of the common *CDKN2A* variants.
5. Assessment of clinical characteristics of *CDKN2A*--positive breast cancers in young women.
6. Assessment of *CDKN2A* common variants and multi-organ cancer risk.
7. Assessment of *XPD* common variants and their association with melanoma and breast cancer risk.
8. Assessment of *MC1R* common variants, *CDKN2A* and their association with melanoma and breast cancer risk.

1. Risk of malignancies of various site of origin in relatives of malignant melanoma patients from families with strong cancer familial aggregation

(based on Debniak T, Gorski B, Cybulski C, Jakubowska A, Kurzawski G, Kladny J, Zaluga E, Fiedorowicz J, Debniak B, Lubinski J. Increased risk of breast cancer in relatives of malignant melanoma patients from families with strong cancer familial aggregation. Eur J Cancer Prev 2003; 12: 241-245)

Population screening for familial aggregation of malignancies performed recently by our centre for the West Pomeranian region of Poland (population 1.5 m) has shown that among families with strong aggregation of tumours the most frequently diagnosed situation is the occurrence of cancer familiar aggregations with unknown pathogenetic background (CFA). CFA do not match pedigree/clinical criteria of any known cancer family syndromes and they are characterized by occurrence of malignancies of various sites of origin among at least three first-degree relatives. It is important to perform analyses to clarify the background of the CFA in order to predict their clinical behaviour, and eventually discern how to defeat them.

One of the tumours that can occur in families with CFA is malignant melanoma (MM). It has been shown that constitutional BRCA1 mutations lead to familial cancer aggregations of not only breast/ovarian cancers but also MM [1]. In addition, it has been suggested that in some families MM can be caused by a predisposition to tumours of various sites [2-4].

The aim of this study was to evaluate the risk of occurrence of malignancies of various sites in patients with MM and their first-degree relatives from families with CFA.

Materials and methods

The pedigree and clinical data of a series of 175 consecutive patients with MM who underwent surgery at the Regional Oncology Hospital in Szczecin in the period from 1997 to 2001 were reviewed in order to select cases with histologically confirmed MM of the skin and family history of at least two more first-degree relatives affected by malignancies of various site of origin (MM/CFA). Families with two or more MM cases among first-degree relatives were considered as familial melanomas and excluded from this study.

We identified 51 families with MM/CFA and analyzed the tumour spectrum and age at diagnosis of malignancies in these families, and compared it to the general Polish population.

The study was approved by the ethics board of the Pomeranian Medical University in Szczecin.

Results

Analysis of distribution of age at diagnosis of MM from MM/CFA families revealed two peaks of occurrence of melanoma cases at the ages 45-50 and 65-70 years. Thus, we identified a subgroup of 22 MM/CFA families with MM diagnosed before 55 years with a total number of 75 malignancies among relatives of patients with MM; and a subgroup of 29 MM/CFA families with MM diagnosed after 55 and with a total number of 78 malignancies among relatives of patients with MM.

Evaluation of the tumour spectrum in these families revealed an increased proportion of breast cancers: 17.52% in the subgroup of ≤55 MM/CFA families, 12.15% in the subgroup of >55 MM/CFA families.

We also observed an increased proportion of liver cancer, CSU (cancer site unknown) and leukaemia.

Evaluation of the mean age at diagnosis revealed that breast cancers appear at a younger age in comparison with the general population; mean age of diagnosis in ≤55 MM/CFA families was 48.47 years (Table 1).

Statistical analyses revealed a higher than expected observed frequency of occurrence of breast cancers

Table 1. Proportion and age at diagnosis of malignancies of various site of origin in relatives of MM patients from MM/CFA families

Tumour site	≤55MM/CFA families			>55MM/CFA families			General population[1]	
	frequency (%)	odds ratio	mean age at diagnosis	frequency (%)	odds ratio	mean age at diagnosis	frequency (%)	mean age at diagnosis
breast	17 (17.52)[3]	2.14	48.47[3]	13 (12.15)	–	50.85	8.96	58.5
≤50 years	10 (10.3)[3]	3.71	–	8 (7.5)[3]	2.61	–	2.77	–
>50 years	7 (7.2)	–	–	5 (4.7)	–	–	6.19	–
lungs	11 (11.34)	–	61.13	10 (9.34)[3]	–	62.23	19.15	64
colon	6 (6.19)	–	65.4	9 (8.41)	–	67.3	9.68	62
stomach	3 (3.09)	–	61.9	6 (5.60)	–	61.4	5.95	67
liver	4 (4.12)	–	66.9	6 (6.54)[3]	–	67.7	1.77	67
skin[4]	3 (3.09)	–	61.8	5 (4.67)	–	62.1	4.47	67.6
leukaemia	3 (4.12)	not done		0	not done		1.95	not done
CSU[2]	12 (12.38)[3]	not done		8 (7.47)[3]	not done		0.42	not done

[1]general Polish population (Zatoński W, Tyczyński J. Nowotwory w Polsce w 1999. Krajowy rejestr nowotworów, Warszawa 2001)
[2]11 cases of ovarian, uterine or cervical cancer 6.1%, site of origin not confirmed
[3]$p<0.05$, statistically significant
[4]skin cancers

(OF 10.48% versus EF 4.54%) in MM/CFA families ≤55 MM/CFA. Related risk of occurrence of breast cancers was especially increased in ≤55 MM/CFA families – 3.256 (Table 2).

Summary of the results

We analysed tumour spectrum and age at diagnosis of malignancies in 51 families with MM/CFA. Families of patients with MM diagnosed <56 years of age showed an increased proportion of breast cancer (OF 10.6%, EF=4.3%, mean age of diagnosis 48.5 years) and a higher proportion of diagnosis of breast cancer in younger age (<50 years of age, OR=3.7; RR=3.3).

2. Germline mutation and large deletion analysis of the *CDKN2A/ARF* genes in families with multiple melanomas and in families with an aggregation of malignant melanoma and breast cancer

(based on Debniak T, Gorski B, Scott RJ, Cybulski C, Medrek K, Zowocka E, Kurzawski G, Debniak B, Kadny J, Bielecka-Grzela S, Maleszka R, Lubinski J. Germline mutation and large deletion analysis of the *CDKN2A* and *ARF* genes in families with multiple melanoma or an aggregation of malignant melanoma and breast cancer. Int J Cancer 2004; 110: 558-562)

The results of previous studies show increased risk of malignant melanoma (MM) in families with multiple members affected by disease and in families characterized by the constellation of breast cancer and MM. The reason for that may be shared molecular base of breast cancer and MM, like for example injury of the *CDKN2A* gene. In the literature, there are some differences concerning the frequency of constitutional mutations of the *CDKN2A* gene in carriers with familiar aggregation of MM in different populations [5-8]. In Polish familial MM (FMM), no *CDKN2A* mutations have been detected to date [9].

CDKN2A shares exons 2 and 3 with another gene, *ARF*, which has also been implicated in the pathogenesis of MM [10]. Hewitt et al. described a germline mutation of *ARF* in patients affected with melanoma or breast cancer from a family with multiple melanomas and breast cancers [11]. No studies examining *ARF* in families with aggregations of MM and breast cancer have been reported.

Methods used to screen for mutations in *CDKN2A/ARF* are usually PCR-based and focus on the detection of small sequence alterations, such as point mutations, small deletions and insertions. Only a few authors have searched for large *CDKN2A/ARF* deletions or rearrangements [6, 12]. A novel method to analyze

Table 2. Results of statistical analyses (expected and observed frequency, related risk of occurrence of malignancies of various site of origin) in MM/CFA families

Tumour site[1]	Observed frequency (OF %)	Expected frequency (EF %)	Relative risk (RR, CI 95%)
breast[2]			
≤55MM/CFA	14[2] (10.6)	5.68 (4.3)	3.256 (CI 95% 0.95–6.8)[4]
>55MM/CFA	13[2] (9.8)	11.35 (8.6)	1.145 (CI 95% 0.54–2.45)
lungs			
males	17 (5.6)	12.57 (4.1)	1.352 (CI 95% 0.63–2.934)
females	4 (1.3)	2.95 (0.9)	1.358 (CI 95% 0.258–7.678)
colon			
males	8 (2.6)	5.17 (1.7)	1.547 (CI 95% 0.47–5.306)
females	7 (2.2)	4.59 (1.5)	1.526 (CI 95% 0.427–5.723)
stomach			
males	5 (1.6)	3.11 (1.0)	1.609 (CI 95% 0.344–8.232)
females	4 (1.3)	2.59 (0.8)	1.543 (CI 95% 0.278–9.577)
liver			
males	4 (1.3)	2.24 (0.7)	1.786 (CI 95% 0.301–12.53)
females	6 (1.9)	2.50 (0.8)	2.396 (CI 95% 0.497–13.83)
skin[3]			
males	5 (1.6)	3.41 (1.1)	1.465 (CI 95% 0.326–7.06)
females	3 (1.0)	3.88 (1.2)	0.773 (CI 95% 0.137–4.107)

[1]breast cancer was studied in two separate groups, the remaining tumours in one group (51 families with MM/CFA)
[2]women only, 3 cases of bilateral or metachronic breast cancer and 1 case of breast cancer in a man were excluded from further study
[3]skin cancer (basocellular or spinocellular)
[4]$p<0.05$

large genomic alterations, multiplex ligation-dependent probe amplification (MLPA), has been described, enabling rapid and sensitive screening for the identification of genomic rearrangements [13, 14].

To determine whether the *CDKN2A* and *ARF* genes are involved in Polish FMM and aggregations of breast cancer and MM, we applied genomic sequencing and MLPA analysis to the molecular characterization of these genes in DNA samples obtained from FMM patients and from CFA families with MM and breast cancer.

Materials and methods

Patients

We examined 3 groups of families: group 1 (FMM cases), MM probands from 16 families with at least 2 first-degree relatives affected with MM registered at the Hereditary Cancer Centre in Szczecin; group 2 (44 cases), probands diagnosed with MM and having at least 2 first-degree relatives, one affected with breast cancer and the other diagnosed with either breast cancer or a malignancy at a different site; group 3 (22 cases), family defined by the index patient being diagnosed with breast cancer, a first-degree relative with melanoma and a family history of at least one more first-degree relative affected by any malignancy.

For restriction fragment length polymorphism (RFLP) analysis, DNA samples from 200 healthy individuals selected at random by family doctors from the city of Szczecin (controls) were used.

Methods

Genomic DNA was prepared from peripheral blood leukocytes [15]. Purified PCR products were sequenced

directly using the Big-Dye Terminator, version 3.0 (Perkin-Elmer, Foster City, CA), sequencing reaction with the same forward primers used previously for exonic amplification of the *CDKN2A* and *ARF* genes. Sequencing reaction products were separated and analyzed on an ABI 377 DNA Sequencer (Perkin-Elmer).

For the analysis of large deletions and insertions, the SALSA P024 *CDKN2A*/2B (9p21) was used. After PCR amplification of exon 2 of *CDKN2A*, products were digested with restriction enzymes BseLI and CFR42I (both from MBI Fermentas, Vilnius, Lithuania).

The study was approved by the ethics board of the Pomeranian Medical University in Szczecin.

Results

In group 1, there were 16 probands screened for mutations in the *CDKN2A/ARF* genes. From this set of families, there were 2 cases identified, one of which harboured a common polymorphism (A148T) and the other a previously unidentified missense change (R99G).

In group 2, of 44 probands, 3 patients (6.8%) were identified who harboured the same common polymorphism as defined above. Only one case in group 3 harboured the A148T change. In the control group, there were 7 cases of 200 probands (3.5%).

There were no changes in exon 1 of the *ARF* gene from any of the probands of the 3 groups. However, the R99G change identified in *CDKN2A* translates to a P144R change in the *ARF* expressed sequence, both of which may result in a functional change in activity. The rare variant identified in the FMM proband was further analyzed in 200 individuals who had not been reported to be affected by any malignancy. The results revealed an absence of the R99G change in the control population, suggesting that it is associated with disease risk.

The presence of large deletions or insertions was evaluated using the MLPA assay, and in none of the 3 groups were gross rearrangements of the *CDKN2A/ARF* genes observed.

Summary of the results

From 16 families with strong aggregations of MM and from 66 families with breast cancer and the other diagnosed with either breast cancer or with strong aggregations of malignancy at a different site, only 6 families with *CDKN2A* constitutional mutations were diagnosed. Two different changes in the *CDKN2A* gene were detected – A148T variant was found in 5 families, and R99G was found in one family.

3. *CDKN2A* common variants and their association with melanoma risk

(based on Debniak T, Scott RJ, Huzarski T, Byrski T, Rozmiarek A, Debniak B, Zaluga E, Maleszka R, Kladny J, Gorski B, Cybulski C, Gronwald J, Kurzawski G, Lubinski J. *CDKN2A* common variants and their association with melanoma risk: a population-based study. Cancer Res 2005; 65: 835-839)

There have been 77 causative *CDKN2A* variants listed in the international melanoma mutation database, eMelanoBase. *CDKN2A* (OMIM 60160) was the first to be associated with MM risk and is regarded as the major MM susceptibility gene. 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 or disease progression. However, one study indicates that there is no overrepresentation of the Nt500c>g and the Nt540c>t variants in the melanoma population [16-18]. Based on the results of the previous paper, it was shown that 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 3.5% of the population. Functional studies suggest this variant is a polymorphism, which seems to have no major affect on p16 function [19, 20]. Previous studies have shown that the A148T polymorphism is in linkage disequilibrium with the promoter polymorphism P-493, which has been shown to affect gene expression [21, 22]. Nevertheless, the A148T change has been found to be overrepresented in melanoma kindreds (3%) in comparison to the general population (1.8%) [18].

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

Materials and methods

Patients

The unselected case group consisted of 471 patients with MM, comprising: a) 301 unselected patients with MM diagnosed in northwestern Poland (Szczecin,

Gorzów Wlkp, Zielona Góra); b) 80 unselected consecutive MM cases diagnosed in northeastern Poland (Białystok); c) 90 unselected consecutive MM cases diagnosed in southwest Poland (Opole).

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.

Methods

The A148T variant was analyzed by RFLP PCR; PCR products were digested with the SacII enzyme. The Nt500c>g and the Nt540c>t variants were analyzed by RFLP PCR; PCR products were digested with the AvaI and HaeIII enzymes.

In cases positive in RFLP PCR, DNA samples were sequenced to confirm the presence of the mutation.

The study was approved by the ethics board of the Pomeranian Medical University in Szczecin.

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%. There were no large differences in the frequencies of the Nt500c>g alleles and in the frequencies of the Nt540c>t alleles in the two control populations (Table 3). 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.

In the melanoma population under study the frequency of the A148T variant was significantly greater than that observed in the control population. There were no large differences in the frequencies of the Nt500c>g alleles or in the frequencies of the Nt540c>t alleles (Table 3).

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. 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). The mean age of A148T carriers was 53 years and the mean age of non-carriers was 55 years; this was not statistically significant (P=0.632).

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 non-carriers. 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 non-carrier 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 non-carriers were studied to determine the proportion of samples that were linked. The results revealed that all A148T heterozygous carriers were heterozygous A/T carriers at position P-493 and all A148T non-carriers were A/A homozygous carriers at position P-493.

Summary of the results

From 471 unselected patients with MM, the A148T change was found in 7% of patients, and the Nt500c>g and Nt540c>t variants were found in 23.6% and in 20% of patients, respectively. All estimate changes were significantly greater in the case group than those observed in the control population. Statistically significant data show that the A148T (OR=2.5) variant seems to be associated with an increased risk of developing MM, insignificantly higher in cases diagnosed <50 years of age (OR=3.4). There were no substantial differences in MM diagnosed in middle age, among carriers and non-carriers of A148T change.

4. Breast cancer risk among carriers of the common *CDKN2A* variant

(based on Debniak T, Gorski B, Huzarski T, Byrski T, Cybulski C, Mackiewicz A, Gozdecka-Grodecka S, Gronwald J, Kowalska E, Haus O, Grzybowska E, Stawicka M, Swiec M, Urbanski K, Niepsuj S, Wasko B, Gozdz S, Wandzel P, Szczylik C, Surdyka D, Rozmiarek A, Zambrano O, Posmyk M, Narod SA, Lubinski J. A common variant of *CDKN2A* (p16) predisposes to breast cancer. J Med Genet 2005; 42: 763-765)

Candidate genes for cancers of a particular site may be selected because they are known to predispose to malignancies of other organs, or because they are mutated somatically in the cells from the cancer of interest. It is possible that missense variants of genes for

Table 3. Frequencies of *CDKN2A* variant alleles in cases and controls

Groups	A148T	OR 95% CI	Nt500c>g	OR 95% CI	Nt540c>t	OR 95% CI
newborns	0 (0%) A/A	–	6 (1.2%) G/G	–	4 (0.8%) T/T	–
(n=500)	14 (2.8%) G/A	–	88 (17.6%) G/C	–	62 (12.4%) C/T	–
	486 (97.2%) G/G	–	406 (81.2%) C/C	–	434 (86.8%) C/C	–
adults	0 (0%) A/A	–	9 (1.3%) G/G	–	3 (0.4%) T/T	–
(n=710)	21 (2.95%) G/A	–	143 (20.1%) G/C	–	83 (11.7%) C/T	–
	689 (97.05%) G/G	–	558 (78.6%) C/C	–	625 (88%) C/C	–
total	0 (0%) A/A	–	15 (1.2%) G/G	–	7 (0.6%) T/T	–
controls	35 (2.89%) G/A	–	229 (18.9%) G/C	–	145 (12%) C/T	–
(n=1210)	1175(97.1%)GG	–	966 (79.8%) C/C	–	1058(87.4%)C/C	–
	Allel A 1.5%	–	Allel G 10.7%	–	Allel T 6.6%	–
MM*	0 (0%) A/A	–	8 (1.7%) G/G	1.4[4] 0.6–3.3	3 (0.6%) T/T	1.1[4] 0.3–4.3
(n=471)	33 (7%) G/A	2.5[1] 1.5–4.1	103 (21.9%) G/C	1.2[4] 0.9–1.6	64 (13.5%) C/T	1.2[4] 0.8–1.6
	438 (93%) G/G	0.4[1] 0.2–0.6	360 (76.4%) C/C	0.8[4] 0.6–1.1	404 (85.7%) C/C	0.9[4] 0.6–1.2
	Allel A 3.5%	2.5[1] 1.5–4.0	Allel G 12.6%	1.2[4] 0.9–1.5	Allel T 7.4%	1.1[4] 0.9–1.5
melanoma	0 (0%) A/A	–	1 (0.6%) G/G	0.5[4] 0.1–3.6	2 (1.2%) T/T	2.0[4] 0.4–9.8
≤50	16 (9.3%) G/A	3.4[2] 1.9–6.4	32 (18.6%) G/C	1.0[4] 0.6–1.5	21 (12.2%) C/T	1.0[4] 0.6–1.7
(n=172)	156 (90.7%) G/G	0.3[2] 0.2–0.5	139 (80.8%) C/C	1.1[4] 0.7–1.6	149 (86.6%) C/C	0.9[4] 0.6–1.5
	Allel A 4.7%	3.3[2] 1.8–6.1	Allel G 9.9%	0.9[4] 0.6–1.3	Allel T 7.3%	1.1[4] 0.7–1.7
melanoma	0 (0%) A/A	–	7 (2.3%) G/G	1.9[4] 0.8–4.7	1 (0.3%) T/T	0.6[4] 0.1–4.7
>50	17 (5.7%) G/A	2.0[3] 1.1–3.7	71 (23.7%) G/C	1.3[4] 1.0–1.8	43 (14.4%) C/T	1.2[4] 0.9–1.8
(n=299)	282 (94.3%) G/G	0.5[3] 0.3–0.9	221 (73.9%) C/C	0.7[4] 0.5–0.9	255 (85.3%) C/C	0.8[4] 0.6–1.2
	Allel A 2.8%	2.0[3] 1.1–3.6	Allel G 14.2%	1.4[4] 1.0–1.8	Allel T 7.5%	1.2[4] 0.8–1.6

*unselected malignant melanomas;
[1]p=0.0003, [2]p=0.0002, [3]p=0.0315, [4]p≥0.05 – not statistically significant

which truncating mutations are clearly pathogenic may also be deleterious, but with reduced penetrance. In this situation the association may be overlooked unless large numbers of cancers are studied. The *CDKN2A* (OMIM 600160) gene is a tumour suppressor gene that is involved in susceptibility to malignant melanoma and has also been implicated in familial pancreatic cancer [23]. The p16 protein is a cyclin-dependent kinase inhibitor that suppresses cell proliferation and is expressed in a wide range of tissues, including the breast, and in breast cancers [24]. In a previous study [reference], it was shown that patients with the A148T variant have higher predisposition to MM (an alanine to threonine substitution at codon 148) (OR=2.5; p=0.0003). It has been suggested by Borg and colleagues that protein truncating *CDKN2A* mutations predispose women to breast cancer in the context of a syndrome of melanoma, pancreatic cancer and breast cancer [22]. They observed eight cases of breast cancer in Swedish families, compared with the 2.1 expected (p=0.002). There are few other data on this topic. Ghiorzo et al. observed a non-significant excess of breast cancers in seven melanoma families with *CDKN2A* mutations (OR=1.9; 95% confidence interval (CI), 0.4 to 5.6) [25]. Somatic *CDKN2A* mutations have not been well studied in breast cancers, but silencing of *CDKN2A* through methylation appears to be a relatively common way of inactivating this tumour suppressor gene in the breast [26]. Furthermore, deletion or loss of heterozygosity at the *CDKN2A* locus (9p21) is relatively common in breast cancers [27].

To establish whether this common missense variant of *CDKN2A* predisposes to breast cancer we undertook an association study on 4209 cases of breast cancer and 3000 ethnically matched controls from Poland.

Materials and methods

During 1997-2003 in 18 treatment centres throughout Poland family history and DNA samples were obtained from 3318 patients with breast cancer diagnosed before the age of 51. In a companion study, seven of these centres also provided data on unselected breast cancer in 891 cases diagnosed above the age of 50 between 2000 and 2002.

The control group comprised 2000 newborn and 1000 adults from the region of Szczecin unselected for cancer family history.

The study was approved by the ethics board of the Pomeranian Medical University in Szczecin.

Laboratory methods

The A148T variant was analyzed by PCR-RFLP. PCR products were digested with the SacII enzyme. The presence of the A148T change was confirmed by direct DNA sequencing.

Results

The A148T variant was detected in 105 from 3000 cases from the control group (3.5%), 5.1% of breast cancer patients diagnosed at age 50 or below and in 4.5% of cases diagnosed at age 51 and above (Table 4). For women diagnosed below age 51 the odds ratio was 1.5 (p=0.002). For women diagnosed over age 50 the odds ratio was modest and non-significant (OR=1.3; p=0.2). In the small group of patients diagnosed with breast cancer at age 30 and under, the prevalence of the *CDKN2A* variant was 12.1% and the association was much stronger (OR=3.8; p=0.0002).

Among the 3318 women diagnosed with breast cancer under age 50, 692 had a family history of a first- or second-degree relative with breast cancer. For the familial and non-familial cases there were no differences observed in frequencies of the A148T variant.

However, the frequency of the A148T alleles was similar in the newborn population (3.5%) and the adult population (3.6%). The allele was equally frequent among males (3.5%) and females (3.6%) and among controls recruited from Szczecin (3.5%) and from elsewhere in Poland (3.6%).

Summary of the results

We have shown that the A148T allele of the *CDKN2A* gene is overrepresented in a population of 3318 unselected patients with breast cancer under age 50, and also in 891 unselected patients with breast cancer at age 50 and above. The statistical significant differences concern risk of breast cancer obtained only in the group of patients with cancer diagnosed in young age (<51 years of age, OR=1.5).

5. Clinical characteristics of *CDKN2A*--positive breast cancers in young women from Poland

(based on Debniak T, Cybulski C, Gorski B, Huzarski T, Byrski T, Gronwald J, Jakubowska A, Kowalska E, Oszurek O, Narod SA, Lubinski J. *CDKN2A*-positive breast cancers in young women from Poland. Breast Cancer Res Treat 2006)

The results of previous papers suggest that the frequent A148T change in the *CDKN2A* gene is associated with higher frequency not only of MM but also of breast cancer in young age (≤50 years of age).

To investigate the contribution of the A148T variant to early-onset breast cancer, and to establish the characteristic features of these cancers, we studied 3069 early-onset breast cancer cases and 3439 population controls.

Materials and methods

The case group consisted of 3267 cases with breast cancer diagnosed under 51 years of age, unselected in relation to family history. Among these, 198 women carried one of the three Polish founder BRCA1 mutations (4153delA, 5328insC, C61G) and were excluded from the present study. Information was recorded on age at diagnosis, stage, grade and lymph-node status, oestrogen-receptor status, multicentricity and bilaterality. The medical record and pathology report were reviewed locally by the physician associated with the study.

Table 4. Frequencies of *CDKN2A* variant alleles in breast cancer patients

Age	Number tested	Mutation positive	Prevalence [%]	Odds ratio	p
≤50 years					
20–30	66	8	12.10	3.8	0.0002
31–40	582	24	4.10	1.2	0.46
41–50	2670	136	5.10	1.5	0.003
total	3318	168	5.10	1.5	0.002
>50 years					
51+	891	40	4.50	1.3	0.17

The control group consisted of 2051 newborn children from hospitals throughout Poland, and 1442 healthy adults unselected for cancer family history.

The study was approved by the ethics board of the Pomeranian Medical University in Szczecin.

Results

An A148T variant was identified in 157 of 3069 women with breast cancer in young age (5.1%; OR=1.4; 95% CI 1.075–1.725; P=0.012).

The characteristics of breast cancer cases in the 157 women with *CDKN2A* variant were compared with non-carriers (Table 5). The mean age of diagnosis in women with an A148T variant was similar to that in the non-carriers cases. The distribution of histological types was similar in cases and controls. However, carriers of A148T variant were more likely to be diagnosed with intraductal cancers (DCIS) with micro-invasion than were non-carriers (14.8% vs. 8.5%; P=0.035). Lobular cancers were less frequent among carriers when compared to non-carriers (16.6% vs. 1.3%), although this difference was not significant (P=0.58).

Carriers and non-carriers were similar with respect to tumour size; there were no significant differences between the groups (Table 5). Carriers and non-carriers were similar with respect to oestrogen-receptor status (67% vs. 64%; P=0.6). Bilateral tumours were equally common in both subgroups (3.6% vs. 3.4%; P=0.9), multicentric cancers were also not over-represented in any of the groups (20% vs. 18%; P=0.6).

There was no difference in cancer family history between carriers and non-carriers of the A148T variant – 7.5% of the carriers were from a family with two or more first-degree relatives with breast cancer versus 9% of the non-carriers (P=0.64).

Summary of the results

The study of 3069 cases with breast cancers certifies that carriers of the A148T variant were slightly more likely to develop intraductal cancers (DCIS) with micro-invasion than non-carriers (14.8% vs. 8.5%; P=0.035). There was no difference between carriers and non-carriers with respect to tumour size, laterality, multicentricity, nodal status, family history or oestrogen receptor status.

6. *CDKN2A* common variant and multi-organ cancer risk

(based on Debniak T, Scott RJ, Huzarski T, Byrski T, Rozmiarek A, Debniak B, Gorski B, Cybulski C, Medrek K, Mierzejewski M, Masojc B, Matyjasik J, Zlowocka E, Teodorczyk U, Lener M, Klujszo-Grabowska E, Nej-Wolosiak K, Jaworowska E, Oszutowska D, Szymanska A, Szymanska J, Castaneda J, van de Wetering T, Suchy J, Kurzawski G, Oszurek O, Narod S, Lubinski J. *CDKN2A* common variant and multi-organ cancer risk – a population-based study. Int J Cancer 2006; 118: 3180-3182)

The *CDKN2A* variant can be found in tumours of many organs, among others head and neck cancer, respiratory malignancies and laryngeal cancer [28-30]. The p16 protein is expressed in a wide range of tissues, and the full range of cancers associated with *CDKN2A* mutations has yet to be determined.

Data from the literature indicate a possible association between *CDKN2A*, A148T and common MM. There is also some evidence to indicate that this variant is associated with breast cancer at younger age. We had also observed that the A148T heterozygous carrier population was more likely to have a first-degree relative with cancer of any type compared to the MM carrier population (p=0.03).

To determine whether this A148T change may be associated with an increased risk of malignancies at various sites of origin, we genotyped a series of 3,583 unselected cancer cases and compared the frequency of the change observed in this population to 3,000 controls.

Materials and methods

Cases were enrolled in the study from hospitals in Szczecin and surrounding counties. Study subjects were unselected for age, sex or family history. In general, more than 75% of patients agreed to take part in the studies. Two control groups were combined. The first group consisted of 2,000 newborn children from ten hospitals throughout Poland. The second control group consisted of 1,000 unselected adults from the region of Szczecin.

The method of examining and verifying the A148T variant was shown previously.

The study was approved by the ethics board of the Pomeranian Medical University in Szczecin.

Results

The A148T variant was detected in 3.5% of Polish controls. The frequency of the alleles was similar in the newborn population (3.4%) compared to the adult population (P=0.83). There was no statistical difference

Table 5. Comparison of *CDKN2A*-positive and *CDKN2A*-negative breast cancer cases

Parameters		p16-positive n=157	p16-negative n=2912	p
age (mean)		44.35	44.34	0.9
age group	20–30	6/157 (3.82%)	51/2912 (1.75%)	0.11
	31–40	21/157 (13.38%)	501/2912 (17.2%)	0.25
	41–50	130/157 (82.80%)	2360/2912 (81.04%)	0.66
histology	"low grade"	32/108 (29.63%)	637/2012 (31.66%)	0.74
	"high grade"	15/108 (13.88%)	303/2012 (15.06%)	0.85
	medullary	6/108 (5.55%)	112/2012 (5.57%)	0.99
	lobular	18/108 (16.66%)	388/2012 (19.28)	0.58
	tubulo-lobular	6/108 (5.55%)	89/2012 (4.42%)	0.75
	DCIS	16/108 (14.81%)	170/2012 (8.45%)	0.04
	other	15/108 (13.88%)	313/2012 (15.56%)	0.74
	no data or unknown	12/108 (11.11%)	225/2012 (11.18%)	0.89
pre-operative chemotherapy	positive	37/151 (24.5%)	675/2012 (24.5%)	0.99
ER	positive	61/91 (67.03%)	1085/1698 (63.9%)	0.58
tumour size	<1 cm	9/99 (9.09%)	185/1767 (10.47%)	0.87
	1–1.9 cm	43/99 (43.43%)	798/1767 (45.16%)	0.76
	2–4.9 cm	46/99 (46.46%)	742/1767 (42%)	0.72
	>5 cm	1/99 (1.01%)	42/1767 (2.38%)	0.73
nodal status	positive	37/100 (37%)	752/1801 (41.75%)	0.4
multi-centricity	present	18/90 (20%)	340/1898 (17.91%)	0.58
laterality	present	5/138 (3.62%)	88/2573 (3.42%)	0.96
family history	positive (+)	11/146 (7.5%)	240/2658 (9%)	0.64

For all comparisons, except age, bilaterality and family history, cases with pre-operative chemotherapy were excluded. Family history refers to a first-degree relative affected with breast cancer

in the *CDKN2A* allele frequencies in the newborns recruited from the Szczecin metropolitan region compared to other Polish cities. There was no evidence that the genotype frequencies of the A148T variant deviated from those expected under Hardy-Weinberg (HWE) for the control groups ($p>0.4$).

The prevalence of the A148T variant was higher in cancer cases than in controls for three of the eleven sites studied (Table 6). The highest odds ratios were observed among lung cancer (OR=2.0) and colon cancer (OR=1.5) cases. For lung the excess was statistically significant (adjusted p value after Bonferroni correction 0.0052), and was non-significant for colorectal cancer (unadjusted p value 0.0423, adjusted p-value 0.5499) (Table 6).

Odds ratios were further analyzed by taking into account the age of disease onset (≤50, >50). There were no significant differences between early-onset lung cancers (5/70; 7.1%) and late-onset cancers (29/427; 6.8%). The A148T prevalence was slightly higher in late-onset colorectal cancers (5.1%) than in early-onset cases (4.9%) (the number of patients with colon cancer diagnosed under age 50 was less (n 5 122), thereby reducing the power of this result).

Table 6. Association between A148T variants and selected types of cancer

Cases	A148T	OR	95% confidence interval	p (adjusted)
controls	0 (0%) A/A	–	–	–
(n=3000)	105 (3.5%) G/A	–	–	–
	2895 (96.5%) G/G	–	–	–
	Allele A frequency 1.75%	–	–	–
bladder	0 (0%) A/A	–	–	–
(n=223)	7 (3.1%) G/A	0.9	0.4105–1.945	0.7764 (n.s.)
	216 (96.9%) G/G	1.1	0.5142–2.436	0.7764 (n.s.)
	Allele A frequency 1.6%	0.9	0.4012–1.876	0.7177 (n.s.)
colon	0 (0%) A/A	–	–	–
(n=724)	37 (5.1%) G/A	1.5	1.012–2.180	0.0423 (0.5499)
	687 (94.9%) G/G	0.7	0.459–0.989	0.0423 (0.5499)
	Allele A frequency 2.6%	1.5	1.008–2.151	0.0443 (0.5759)
stomach	0 (0%) A/A	–	–	–
(n=246)	8 (3.3%) G/A	0.9	0.4461–1.925	0.8384 (n.s.)
	238 (96.7%) G/G	1.1	0.5194–2.241	0.8384 (n.s.)
	Allele A frequency 1.6%	0.9	0.4494–1.916	0.8398 (n.s.)
larynx	0 (0%) A/A	–	–	–
(n=396)	17 (4.3%) G/A	1.2	0.7326–2.088	0.4255 (n.s.)
	379 (95.7%) G/G	0.8	0.4790–1.365	0.4255 (n.s.)
	Allele A frequency 2.1%	1.2	0.7337–2.067	0.4298 (n.s.)
ovary	0 (0%) A/A	–	–	–
(n=340)	12 (3.5%) G/A	1.0	0.5491–1.853	0.9777 (n.s.)
	328 (96.5%) G/G	1.0	0.5396–1.821	0.9777 (n.s.)
	Allele A frequency 1.8%	1.0	0.5520–1.843	0.9779 (n.s.)
lung	0 (0%) A/A	–	–	–
(n=497)	34 (6.8%) G/A	2.0	1.358–3.018	0.0004 (0.0052)
	463 (93.2%) G/G	0.5	0.331–0.736	0.0004 (0.0052)
	Allele A frequency 3.4%	2.0	1.342–2.945	0.0005 (0.0065)
prostate	0 (0%) A/A	–	–	–
(n=348)	13 (3.7%) G/A	1.1	0.5946–1.925	0.8215 (n.s.)
	335 (96.3%) G/G	0.9	0.5194–1.682	0.8215 (n.s.)
	Allele A frequency 1.9%	1.1	0.5972–1.912	0.8231 (n.s.)
kidney	0 (0%) A/A	–	–	–
(n=264)	6 (2.3%) G/A	0.6	0.2788–1.474	0.2915 (n.s.)
	258 (97.7%) G/G	1.6	0.6782–3.586	0.2915 (n.s.)
	Allele A frequency 1.1%	0.6	0.2820–1.477	0.2957 (n.s.)
thyroid	0 (0%) A/A	–	–	–
(n=173)	3 (1.7%) G/A	0.5	0.1528–1.549	0.2129 (n.s.)
	170 (98.3%) G/G	2.1	0.6454–6.545	0.2129 (n.s.)
	Allele A frequency 0.9%	0.5	0.1550–1.556	0.2169 (n.s.)
nonHodgkin	0 (0%) A/A	–	–	–
lymphoma	6 (3.7%) G/A	1.1	0.4585–2.453	0.8909 (n.s.)
(n=162)	156 (96.3%) G/G	0.9	0.4077–2.181	0.8909 (n.s.)
	Allele A frequency 1.9%	1.0	0.4616–2.431	0.8918 (n.s.)
pancreas	0 (0%) A/A	–	–	–
(n=210)	8 (3.8%) G/A	1.1	0.5246–2.273	0.8140 (n.s.)
	202 (96.2%) G/G	0.9	0.4400–1.906	0.8140 (n.s.)
	Allele A frequency 1.9%	1.1	0.5275–2.253	0.8156 (n.s.)

Summary of the results

In the study of 3583 unselected patients with tumours of many organs (other than MM and breast cancer) the A148T variant was detected more often than among patients with lung cancer (6.8%; OR=2.0; P=0.0004 adjusted p value after Bonferroni correction P=0.0052) or colon cancer (5.1%; OR=1.5, P=0.0423; adjusted p value after Bonferroni correction P=0.5499).

7. *XPD* common variants and their association with melanoma and breast cancer risk

(based on Debniak T, Scott RJ, Huzarski T, Byrski T, Masojc B, van de Wetering T, Serrano-Fernandez P, Gorski B, Cybulski C, Gronwald J, Debniak B, Maleszka R, Kladny J, Bieniek A, Nagay L, Haus O, Grzybowska E, Wandzel P, Niepsuj S, Narod SA, Lubinski J. *XPD* common variants and their association with melanoma and breast cancer risk. Breast Cancer Res Treat 2006; 98: 209-215)

There is continuing interest in identifying low-penetrance genes, which are associated with increased susceptibility to common types of cancer. The genetic basis of MM is complex and appears to involve multiple genes. Individuals with the rare inherited syndrome xeroderma pigmentosum (XP) have an approximate 1000-fold increased incidence of skin malignancies, including melanoma [31]. There are several genes associated with XP and these include ERCC2, ERCC3, XP-G and XP-F [31, 32]. The *XPD* (ERCC2) gene product has a dual function in basal transcription and in nucleotide excision repair [33, 34]. In the literature there are four reports evaluating *XPD* gene polymorphisms and melanoma risk. Two of them were performed on a very small number of cases [35, 36], whereas the other two suggested a modest positive association of two single nucleotide polymorphisms in exons 10 (Asp312Asn) and 23 (Lys751Gln) with either melanoma or a subset of older onset cases of melanoma based on 219 and 176 melanoma cases, respectively [37, 38]. Recently the two *XPD* variants (Asp312Asn and Lys751Gln) have also been linked with the occurrence of breast cancer [39, 40]. Interestingly, the positive findings for Asp312Asn in the 223 Finnish unselected breast cancer patients were not repeated in 172 Polish familial cases [41].

To establish whether the *XPD* common variants Asp312Asn and Lys751Gln are associated with increased melanoma or breast cancer risk we performed an association study based on genotyping 426 unselected patients with MM and 1830 consecutive breast cancer cases compared to 1262 geographically matched newborns and 1553 healthy adults.

Additionally we examined the prevalence of Gly156Gly, Leu485Pro and Arg112His variants in *XPD* among 421 unselected melanoma patients as it is unknown whether these SNPs are associated with disease risk.

Materials and methods

The first case group of 471 unselected patients with MM consisted of: a) 301 unselected patients with MM diagnosed in northwestern Poland (Szczecin, Gorzów Wlkp, Zielona Góra); b) 80 unselected consecutive MM cases diagnosed in northeastern Poland (Białystok); c) 90 unselected consecutive MM cases diagnosed in southwest Poland (Opole).

The second case group consisted of cases of consecutive invasive breast cancer diagnosed at 7 treatment centres throughout Poland, unselected for age and family history (including 511 cases in the vicinity of Szczecin). A detailed family history of cancer was ascertained. A total of 2500 incident cases of invasive breast cancer were identified at the 7 different centres during the study period. Of these, 1900 women accepted the invitation to participate in this genetic study (76%). From the total of 1900 enrollees 70 patients could not be genotyped because of poor quality DNA. This brought the total number of breast cancer cases studied to 1830.

The first control group consisted of 1262 geographically matched newborn male and female children collected in the same hospitals from where the melanoma and breast cancer cases were collected.

The second control group consisted of 621 adults from the region of Szczecin unselected for cancer family history.

The third control group consisted of 421 healthy adults matched for sex and age with the melanoma cases and 511 healthy adults from the region of Szczecin matched for sex and age with the breast cancer cases collected from the city of Szczecin.

The *XPD* changes were analyzed by PCR-RFLP, using the enzymes: Psp 14061 for Asp312Asn; PstI for Lys751Gln; TfiI for Gly156Gly; HpaII for Leu485Pro; FauI for Arg112His. DNA sequencing was undertaken on random samples to ensure that all PCR products represented the respective *XPD* alleles.

The study was approved by the ethics board of the Pomeranian Medical University in Szczecin.

Results

There were no significant differences in the allele frequencies of the *XPD* variants in all three control

populations; also among newborns recruited from treatment centres throughout Poland and among males and females there were no significant differences. Since only the newborn group consisted of cases geographically matching the melanoma and breast cancer patients, and as it was larger than the adult control population, further statistical calculations were performed only on this control group.

The expected allelotype distributions for all polymorphisms were in Hardy–Weinberg equilibrium.

We were unable to evaluate an association between melanoma and Arg112His and Leu485Pro since neither of these polymorphisms were represented among the control and subject populations used in this study, suggesting that both changes are very rare in the Polish population.

We found no association with the Lys751Gln and Arg112His genotype and melanoma or breast cancer risk. There were no statistically significant differences in Lys751Gln and Arg112His genotype variant prevalence between early and late-onset cases. There were no major differences in the mean age of diagnosis between subjects carrying the AA, AC or CC genotypes of Lys751Gln change and the AA, AC or CC genotypes of Lys751Gln change.

No association was observed between the Gly156Gly genotype and melanoma risk, for either early or late-onset melanoma. There was no major difference in the mean age of diagnosis between the subjects carrying AA, AC or CC genotypes.

We found an association of the Lys751Gln_CC/Asp312Asn_AA genotype with breast cancer risk (Table 7). The genotype was significantly over-represented among early-onset breast cancer patients (OR=1.5, p=0.0212) and late onset cases (OR=1.5, p=0.0169). No association between compound heterozygous carriers of Lys751Gln and Asp312Asn variants and melanoma risk was observed (Table 7). The genotype Lys751Gln_CC/Asp312Asn_GG [42] is rare in our population (1.7% in MM and 1.6% in the control group).

Subjects carrying Lys751Gln_CC genotype and Gly156Gly_CC genotype were associated with modest increase of melanoma risk (OR=1.4). In a subgroup of late-onset melanoma patients the genotype was more tightly associated with melanoma risk with OR=1.7, $p<0.05$ (Table 8).

There were no significant differences among carriers of Asp312Asn + Gly- 156Gly genotype and the risk of MM among early- and late-onset cases. The highest OR (1.4) and lowest p value (0.088) reached was in the subgroup harbouring the Asp312Asn_AA/Gly156Gly_CC genotype.

Table 7. Lys751Gln/Asp312Asn genotypes in the study and control groups

Lys751Gln	Asp312Asn	Melanoma (n=423)	Newborns (n=1017)	OR (95% CI), p	Breast cancer (n=1713)	OR (95% CI), p
AA	GG	115 (27.2%)	267 (26.3%)	1.0 (n.s.)	494 (28.8%)	1.1 (n.s.)
AA	AG	28 (6.6%)	104 (10.2%)	0.6 (n.s.)	153 (8.9%)	0.9 (n.s.)
AA	AA	2 (0.5%)	16 (1.6%)	0.3 (n.s.)	17 (1%)	0.6 (n.s.)
AC	GG	46 (10.9%)	107 (10.5%)	1.0 (n.s.)	151 (8.8%)	0.8 (n.s.)
AC	AG	131 (31%)	334 (32.8%)	1.0 (n.s.)	559 (32.6%)	1.0 (n.s.)
AC	AA	28 (6.6%)	49 (4.8%)	1.4 (n.s.)	77 (4.5%)	1.0 (n.s.)
CC	GG	7 (1.6%)	20 (2%)	0.8 (n.s.)	22 (1.3%)	0.7 (n.s.)
CC	AG	27 (6.4%)	51 (5%)	1.3 (n.s.)	67 (3.9%)	0.8 (n.s.)
CC	AA	39 (9.2%)	69 (6.8%)	1.4 (n.s.)	173 (10.1%)	1.5 (1.2–2.1) 0.004 (0.016)[1]

[1]p value calculated with the χ^2 test (Bonferroni adjusted value in parantheses)

None of the compound heterozygous genotypes were significantly over-represented among melanoma cases. Carriers of the Lys751Gln_CC/Asp312 Asn_GG/Gly156Gly_CC genotype had OR=1.8 with p=0.5. Carriers of the Lys751Gln_CC/Asp312Asn_AA/Gly156Gly_CC genotype had OR=1.3 with p=0.3. The high p value is due to the small numbers of carriers with all three polymorphisms.

Haplotype frequency analyses showed a statistically significant association in subjects carrying Lys751Gln_C/Gly156Gly_C genotype with modest increase of MM risk (OR=1.2; p=0.007 before Bonferroni correction; p=0.028 after Bonferroni correction). We found an association of the Lys751Gln_C/Asp312Asn_A genotype with breast cancer risk. The genotype was significantly over-represented (OR=1.2; p=0.049 before Bonferroni correction, p=0.2 after Bonferroni correction). There was a similar situation with Lys751Gln_C/Asp312Asn_A/Gly156Gly_C genotype (OR=1.2; p=0.008 before Bonferroni correction, p=0.065 after Bonferroni correction).

Summary of the results

XPD genotype Lys751Gln_CC/Gly156Gly_CC was detected in 15% of 471 unselected malignant melanoma patients and 11% of controls (OR=1.4). Significant over-representation of this genotype was found in late-onset cases (over the age of 50, OR=1.7, p=0.007, p=0.042 after Bonferroni correction).

XPD genotype Lys751Gln_CC/Asp312Asn_AA was detected in 10% of 1830 unselected breast cancer patients and 6.8% of controls. This difference was statistically significant p=0.004 (p=0.016 after Bonferroni correction) and OR=1.5.

8. *MC1R* common variants, *CDKN2A* and their association with melanoma and breast cancer risk

(based on Debniak T, Scott R, Masojc B, Serrano-Fernandez P, Huzarski T, Byrski T, Debniak B, Gorski B, Cybulski C, Medrek K, Kurzawski G, van de Wetering T, Maleszka R, Kladny J, Lubinski J. *MC1R* common variants, *CDKN2A* and their association with melanoma and breast cancer risk. Int J Cancer 2006; 119: 2597-2602)

One of the melanoma low-penetrance susceptibility genes is *MC1R*. The *MC1R* gene (16q24, OMIM 155555) encodes a protein that acts as the receptor for melanocyte-stimulating hormone (MSH). It has been reported that some germline allelic variants of *MC1R*

Table 8. Lys751Gln/Gly156Gly genotypes among melanoma and control groups

Lys751Gln	Gly156Gly	Melanoma (MM) (n=424)	Newborns (n=1052)	95% CI	p	OR
AA	AA	56 (13.2%)	157 (14.9%)	–	n.s.	0.9
AA	CA	70 (16.5%)	182 (17.3%)	–	n.s.	1
AA	CC	19 (4.5%)	62 (5.9%)	–	n.s.	0.7
AC	AA	7 (1.65%)	25 (2.4%)	–	n.s.	0.7
AC	CA	130 (30.66%)	318 (30.2%)	–	n.s.	1
AC	CC	66 (15.57%)	153 (14.6%)	–	n.s.	1.1
CC	AA	2 (0.50%)	2 (0.2%)	–	n.s.	2.5
CC	CA	9 (2.12%)	36 (3.4%)	–	n.s.	0.6
CC	CC	65 (15.3%)	117 (11.12%)	1.044–2.006	0.03 (0.18)	1.4
CC	CC	MM≤50[1] (n=172) 21 (12.2%)	117 (11.12%)	–	n.s.	1.1
CC	CC	MM>50[1] (n=250) 44 (17.6%)	117 (11.12%)	1.170–2.491	0.007 (0.042)	1.7

p value calculated with the ÷2 test (Bonferroni adjusted value in parantheses)
[1]age at onset unknown in two cases of melanoma

gene (Arg151Cys, Arg160Trp, Asp294His) are associated with an increased risk of multiple melanomas (MM) [43-45]. They also act as modifiers of melanoma risk in carriers of *CDKN2A* mutations by increasing disease penetrance in familial melanoma cases. However, the majority of these reports suggesting an association were based on the examination of melanoma-prone families [46, 47]. The risk of MM has only been evaluated in fair-skinned populations of northern European [44, 48, 49] origin and in Mediterranean populations [47, 50-53].

Recently Begg et al. pointed out that *CDKN2A* mutation carriers in the general population have a much lower risk of melanoma than that suggested by estimates obtained from multiple-case families [54]. Similarly, the increase of *CDKN2A* penetrance caused by *MC1R* variants may also be lower in the general population.

To evaluate malignant melanoma risk among carriers of common germline *MC1R* changes the protein coding sequence of this gene was analysed and the frequencies of detected DNA alterations were evaluated among cases and controls. In order to evaluate the phenotypic characterisation of the patient population we included information on family aggregation and clinical data in the analyses.

MC1R has recently been suggested to act on MM risk via non-pigmentary mechanisms [50]. Its variant has been reported to be associated with prostate cancer risk. Since melanoma and breast cancer appear to share some genetic background we also examined the prevalence of the *MC1R* variants among unselected breast cancer patients.

Materials and methods

The sequencing was performed in 40 MM patients from families with at least two diagnosed cases among first-degree relatives.

The association research was performed on two case groups. The first case group consisted of 500 unselected MM patients diagnosed in few centres in Poland. The second study population consisted of 511 prospectively ascertained cases of consecutive invasive breast cancer diagnosed in the city of Szczecin.

The control group consisted of 800 geographically matched newborn male and female children collected in the same hospitals from where the melanoma and breast cancer cases were collected: 421 healthy adults matched for sex and age with the melanoma cases and 511 healthy women from the region of Szczecin matched for sex and age with the breast cancer cases collected from the city of Szczecin.

From the sequencing results 4 common variants were identified: V60L, R151C, R163Q and R160W. The frequency of the 4 *MC1R* variants was identified by RFLP-PCR by use of enzyme: Hha I for R151C; HindIII for R163Q; SacII for R160W. The V60L variant was analyzed using an allele-specific PCR (ASO-PCR).

A separate DNA sample was sequenced to confirm the presence of the mutation.

The study was approved by the ethics board of the Pomeranian Medical University in Szczecin.

Results

Genomic sequencing of the 40 familial cases of MM revealed 9 different SNPs. Four of them (V60L, R151C, R163Q and R160W) were regarded as common variants (they were detected in at least 10% of familial cases) and used in further analyses.

There were no significant differences in the allele frequencies of the *MC1R* variants in the 2 control populations. Since only the newborn group consisted of cases geographically matching the melanoma and breast cancer patients, and as it was larger than the adult control population, further statistical calculations were performed only on this control group.

Haplotype analysis in melanoma patient population

We found a statistically significant association of the R151C variant (p=0.000008; OR 5 2.9, 95% CI 1.82–4.67), the V60L variant (p=0.007; OR 5 1.78; 95% CI 1.2–2.64), the R160C variant (p=0.006; OR 5 1.76; 95% CI 1.75–2.62) and the R163Q variant (p=0.015; OR 5 2.1 95% CI 1.1–3.97) with melanoma risk.

The presence of any of those 4 common *MC1R* variants was significantly higher among patients than in the control population (p=0.0000004).

Additionally the association between carriers of the common A148T variant of *CDKN2A* and the 4 *MC1R* polymorphisms was evaluated. None of the compound heterozygotes were significantly over-represented among any of the melanoma cases (data not shown). Nevertheless, the highest OR (4.2) was observed in patients harbouring the A148T variant in *CDKN2A* and the R151C variant in *MC1R*.

Although the average age at diagnosis of carriers was always 1–2 years lower than among non-carriers, for each of the 4 common *MC1R* variants separately, none of these differences was significant. However, there was a statistically significant difference of almost 6 years (t-test, one-tailed; p=0.039) when comparing the age at diagnosis of compound carriers (average 48.4 years) with non-carriers (average 54.2 years).

There was a significantly higher frequency of melanoma occurrence among first-degree relatives of

carriers of any of the *MC1R* variants in comparison to non-carriers (p=0.03; OR=3.6). Analogously there was a slight increase in the prevalence of all *MC1R* variants among melanoma probands with a first- or second-degree relative affected by melanoma (7/40; 17.5%) when compared to unselected cases (73/474; 15.4%).

There were no significant differences in frequency of breast cancer in first-degree relatives and in patients without changes in the *MC1R* gene (Table 9).

The analysis of the clinical data revealed a significant increase of melanoma occurrence on non-exposed skin areas among carriers of any of the *MC1R* variants (p 5 0.0014; OR 5 2.2). We also observed an almost 5-fold increase of multiplicity of melanoma among carriers of any of the *MC1R* variants. There was no association between those variants and tumour type (Table 10).

Haplotype analysis in breast cancer patient population

We found no association with the *MC1R* variants and breast cancer risk. There were no statistically significant differences in the prevalence of the *MC1R*

Table 9. Occurrence of melanoma and breast cancer among first-degree relatives of MM probands

Feature	R160W		p	95% CI	OR
first-degree relatives affected with	carriers (+) n=85	non-carriers (–) n=373			
melanoma[3]	4 (4.7%)[1]	17 (4.5%)[2]	n.s.	–	–
breast cancer[4]	4 (4.7%)	25 (6.7%)	n.s.	–	–
	R151C				
	carriers (+) n=74	non-carriers (–) n=401			
melanoma	7 (9.5%)	11 (2.7%)	0.0097	1.276–9.061	3.4
breast cancer	5 (6.7%)	25 (6.2%)	n.s.	–	–
	V60L				
	carriers (+) n=78	non-carriers (–) n=354			
melanoma	5 (6.4%)	10 (2.8%)	n.s.	–	2.4
breast cancer	5 (6.4%)	24 (6.7%)	n.s.	–	–
	R163Q				
	carriers (+) n=30	non-carriers (–) n=354			
melanoma	1 (3.3%)	13 (3.6%)	n.s	–	–
breast cancer	2 (5%)	17 (4.8%)	n.s	–	–
	any of four variants positive	none of four variants positive			
	carriers (+) n=245	non-carriers (–) n=149			
melanoma	17 (6.9%)	3 (2.0%)	0.03	1.05–12.60	3.6
breast cancer	16 (6.5%)	8 (5.4%)	n.s	–	–

[1]percentage of carriers with at least one affected first-degree relative
[2]percentage of probands without *MC1R* variants but with at least one affected first-degree relative
[3]number of melanoma cases among first-degree relatives
[4]number of breast cancer cases among first-degree relatives

Table 10. Clinical data comparison of carriers versus non-carriers of *MC1R* variants

Feature	Carriers (+)	Non-carriers (–)	p (95% CI)	OR
		R160W		
multiplicity[1]	3/85[2] (3.5%)	6/374[3] (1.6%)	n.s.	2.2
localization-skin[4]	2.4[5]	1.3	n.s.	1.8
tumour type[6]	60[7]/20[8]/10[9]/4[10]	58/20/9/5	n.s.	
		R151C		
multiplicity	3/73 (4.1%)	7/402 (1.7%)	n.s.	2.4
localization in skin	2.2	1.4	n.s.	1.5
tumour type	57/21/9/5	54/19/10/6	n.s.	
		V60L		
multiplicity	2/78 (2.6%)	6/355 (1.7%)	n.s.	1.5
localization in skin	2.0	1.4	n.s.	1.4
tumour type	58/22/11/4	57/20/11/3	n.s.	
		R163Q		
multiplicity	1/30 (3.3%)	8/355 (2.2%)	n.s.	1.5
localization in skin	1.9	1.5	n.s.	1.3
tumour type	60/19/10/5	58/18/9/5	n.s.	
	any of four variants positive	none of four variants positive		
multiplicity	8/254 (3.1%)	1/149 (0.7%)	n.s.	4.8
localization in skin	2.2	1.0	0.0014 (1.35–3.63)	2.2
tumour type	61/21/8/5	60/20/9/4	n.s.	

[1]synchronic or metachronic tumours
[2]number of multiple melanoma cases/total number of *MC1R* variant carriers
[3]number of multiple melanoma cases/total number of patients without *MC1R* variants
[4]regions of exposed skin: head, nape, neck, forearms, palms, shanks, feet; regions of unexposed skin: trunk, thighs, arms, buttocks
[5]unexposed to exposed skin ratio – number of melanomas in unexposed skin divided by number of melanomas in exposed skin
[6]types of melanoma: a) SSM-superficial spreading melanoma; b) NM-nodular melanoma; c) LM-lentigo melanoma; d) ALM-acral lentigo melanoma
[7]SSM percentage
[8]NM percentage
[9]LM percentage
[10]ALM percentage

variants between early and late onset cases. None of the compound heterozygotes (2 or more *MC1R* changes, also MCIR alterations with the A148T *CDKN2A* variant) were significantly over-represented among cases (data not shown).

Summary of the results

Among 500 unselected MM the four variants of *MC1R* gene were found more frequently in the control group: R151C (15.4% MM; OR=2.9; p=0.000 008), V60L (18.2% MM; OR=1.8; p=0.007), R160C (19.3%; OR=1.8; p=0.006) and R163Q (7.8%; OR=2.1; p=0.015). In carriers of the mentioned changes MM appears with higher frequency on non-exposed skin areas (p=0.0014). Mentioned melanoma appears with higher frequency among first-degree relatives of carriers of mentioned changes of the *MC1R* gene (p=0.0097). We certify a statistically significant difference of almost 6 years in comparing the age at diagnosis of compound carriers of variants of the *MC1R* gene.

The highest risk of affected MM was observed among carriers of A148T and R151C variant (OR=4.2); there were no statistically significant data for subgroups of carriers with two or more changes in the *MC1R* gene or carriers with A148T and *MC1R* genes.

Evaluation of 511 unselected breast cancer patients revealed no association with the *MC1R* variants and breast cancer risk.

General conclusions

The outcomes of our various studies lead us to the following conclusions.

Systematic breast surveillance beginning at the age 35–40 years should be considered as an option for women from families with strong cancer familial aggregation of malignancies of various site of origin and for women with malignant melanoma diagnosed under 56 years of life. The outcome of such surveillance would need to be monitored in a research setting to determine its clinical value.

In the Polish population *CDKN2A* and *ARF* do not contribute significantly to either familial melanoma or malignant melanoma within the context of a cancer familial aggregation of disease with breast cancer. Therefore, in this population, there appears to be no strong indication for *CDKN2A* and *ARF* testing in these familial cases.

The common variant of the *CDKN2A* gene A148T, previously regarded as non-pathogenic, may predispose to malignant melanoma, early-onset breast cancer and lung cancer. A148T is significantly over-represented in women with early-onset breast cancer and may predispose to intraductal cancers (DCIS) with micro-invasion. It seems thus justified to propose mammography, in a research setting, beginning at the age of 35 as an option for carriers of the A148T variant.

Compound carriers of common *XPD* variants may be at slightly increased risk of breast cancer or late-onset malignant melanoma.

Common recurrent variants of the *MC1R* gene (V60L, R151C, R163Q and R160W) may predispose to malignant melanoma.

In general, the establishment of surveillance protocols proposed as an option for carriers of common alterations in *CDKN2A*, *XPD* or *MC1R* variants requires additional studies.

All the above-mentioned variants are low-penetrant changes characterized by a negative cancer family history. Thus, the use of DNA tests is the only way to find carriers of these changes with increased risk of tumour development. Identification of low penetrance DNA alterations may be very important if the simultaneous presence of such alternations and/or combinations with external risk factors in a carrier would add up to a clinically significant high risk of tumour development. It will be a big challenge to study the possible cumulative effects on cancer risk of the many different combinations of low-penetrance mutations. Studies of this kind will need very large cohorts to establish reliable risk estimates.

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