MCIR common variants, CDKN2A and their association with melanoma and breast cancer risk

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We sought to examine the association between MCIR variants and the risk of melanoma and breast cancer in Polish population. We also determined the prevalence of compound heterozygous carriers of MCIR and CDKN2A (A148T) variants. We examined 500 unselected melanoma cases, 511 consecutive invasive breast cancer patients, 800 newborns, 421 healthy adults matched for sex and age with the melanoma cases and 511 healthy women matched for sex and age with the breast cancer cases. A statistically significant association of all 4 MCIR variants with the melanoma risk was found. For the R151C variant p value was 0.000008 and odds ratio 2.9; for the V60L variant p value was 0.007 and OR 1.78; for the R160C p was 0.006 and OR 1.76; for the R163Q p was 0.015 and odds ratio 2.1. None of the compound heterozygotes were significantly over-represented among any of the melanoma cases, the highest OR (4.2) observed in patients harbouring the A148T variant. Positive association of all 4 MCIR variants with the melanoma risk was found. The risk of disease seems to be increased additively for patients harbouring also the CDKN2A variant. The association between MCIR and MM in other populations is still unknown. We also found no population-based reports about the association between compound heterozygous carriers of CDKN2A and MCIR variants and the melanoma risk. Thus herein we sought to examine the association between MCIR variants and MM risk in Polish population. We also tried to determine the prevalence of compound heterozygous carriers of MCIR and CDKN2A (A148T) variants in the same general population.

MCIR has recently been suggested to act on MM risk via non-pigmentary mechanisms. Its variant has been reported to be associated with prostate cancer risk. Since many low-penetrant genes appear to be associated with multi-organ cancer susceptibility and melanoma and breast cancer appear to share some genetic background we also examined the prevalence of the MCIR variants among unselected breast cancer patients. To evaluate the phenotypic characterisation of the patients population we included information on family aggregation and clinical data in the analyses.

Key words: MCIR; CDKN2A; melanoma; breast cancer

There is continuing interest in identifying low-penetrance genes which are associated with an increased susceptibility to common types of cancer, including malignant melanoma. However, the identification of genes that are associated with modest disease penetrance requires very large association studies. Furthermore not all populations harbour carriers at the same frequency. One of the melanoma low-penetrance susceptibility genes is MCIR. The MCIR gene (16q24, OMIM 1555555) encodes a protein that acts as the receptor for melanocyte-stimulating hormone (MSH). It has been reported that some germline allelic variants of MCIR gene (Arg151Cys, Arg160Trp, Asp294His) conferred a fair skin/red haired phenotype, which has been associated with an increased risk of multiple melanomas (MM). The same variants, independently of skin type, are associated with an increased MM risk. The 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. Recently Begg et al. pointed 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. It is obvious that such an effect probably also includes MCIR. Similarly, the increase of CDKN2A penetrance caused by MCIR variants may also be lower in the general population. As mentioned above, to date the prevalence of MCIR variants has been investigated mainly in patients with a suspected hereditary predisposition to MM. The relationship between MCIR and MM in other populations is still unknown. We also found no population-based reports about the association between compound heterozygous carriers of CDKN2A and MCIR variants and the melanoma risk. Thus herein we sought to examine the association between MCIR variants and MM risk in Polish population. We also tried to determine the prevalence of compound heterozygous carriers of MCIR and CDKN2A (A148T) variants in the same general population.

MCIR has recently been suggested to act on MM risk via non-pigmentary mechanisms. Its variant has been reported to be associated with prostate cancer risk. Since many low-penetrant genes appear to be associated with multi-organ cancer susceptibility and melanoma and breast cancer appear to share some genetic background we also examined the prevalence of the MCIR variants among unselected breast cancer patients. To evaluate the phenotypic characterisation of the patients population we included information on family aggregation and clinical data in the analyses.

Patients and methods

Melanoma patients

The unselected case group consisted of 500 MM patients (281 females, 219 males, mean age at onset 54.5 years, age range 20–85), comprising 330 unselected patients with MM (mean age 54.7 years, range 26–78) diagnosed in north-western Poland between 2000 and 2004 (Szczecin, Gorzów Wlkp., Zielona Góra), 80 unselected consecutive MM cases (mean age 53.2 years, range 29–74) diagnosed between 2002 and 2003 in north-eastern Poland (Białystok) and 90 unselected consecutively collected MM cases (mean age 54.1 years, range 34–79) diagnosed between 2002 and 2003 in south-west Poland (Opole).

All MM cases were identified from cancer registries in the 5 cities mentioned above. Participation rates exceeded 75% for Białystok and Opole. In north-western Poland (330 cases regarded as unselected for age, sex or cancer family history) the participa-
tion rate exceeded 75%; in Szczecin (223 cases regarded as unselected consecutive MM) between 50% and 75% of cases were recruited, in Gorzów Wlkp. (47 cases regarded as unselected but non-consecutive) and Zielona Góra (40 cases regarded as unselected but non-consecutive).

Among 500 unselected cases, 58 patients (11.6%) had a first or second degree relative affected with MM of which 15 patients (3.0%) had a first degree relative affected with MM (familial melanoma gene). In 26 unselected cases we were unable to perform molecular analyses due to poor quality of DNA.

Searching for common DNA alterations the entire coding region of MC1R gene was sequenced in 40 familial melanoma cases; 14 of these patients were consecutive cases from the series of 500 unselected melanomas, 26 of them were additional cases selected during genetic counselling at our centre.

Breast cancer patients

The study population includes 511 prospectively ascertained cases of consecutive invasive breast cancer diagnosed in the city of Szczecin. The patients were invited to participate in person during their hospital stay or by mailed invitation. During the interview the goals of the study were explained, informed consent was obtained, genetic counselling was given and a blood sample was taken for DNA analysis. A detailed family history of cancer was ascertained (first, and second-degree relatives included) and a risk factor questionnaire was completed.

Controls

The first 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. Samples of cord blood from unselected infants were forwarded to the study centre in Szczecin.

The second control group consisted of 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.

To ensure the comparability of the control groups, the allele frequencies were computed separately for the adult and neonatal control groups and were compared.

Methods

DNA samples were isolated from peripheral blood or from umbilical cord blood of newborns according to the method of Miller et al.15

Sequencing was performed according to standard procedures using the primers M1RAF (5'-CTG GCA GCA CCA TGA ACA TAA G-3'), M1RAR (5'-AAGGA ACGTGC CAG GTCA C-3'), M1RCF (5'-TGATCAC CTGCGATCC TCA C-3'), M1RCA (5'-TGTTG AGC GCA GTGC CGATG-3') and M1RCR (5'-CTG GCA GCA CCA TGA ACT AA G-3') in the presence of the fluorescent labelled dideoxy-chain terminators16 with the ABI Prism Kit (PE Biosystems), in a model 373 automated DNA sequencer (PE Biosystems), in a model 373 automated DNA sequencer (PE Biosystems).

From the sequencing results 4 common variants were identified: V60L, R151C, R163Q and R160W. The frequency of the 4 MC1R variants were assessed as follows:

The R151C variant was identified by RFLP-PCR by use of Hba I (Fermentas) and primers R151CFun (5'-TCTG TGCTTCCCTG AGC GACATC-3') and R151CRN (5'-CCA GCA TGT GGA CGT ACA G-3'). The wild type PCR product was digested with the Hhal enzyme whereas the mutated variant was not.

The R163Q variant was analyzed by allele-specific PCR assay and primers V60L1 (5'-TTGGT GGA GAACGCGG CTGG GAAG-3'), V60L2F (5'-TTGGT GGA GAACGCGG CTGG GAGG-3'), V60L2R (5'-TTGGT GGA GAACGCGG CTGG GAAG-3'), 108f (GCTA AT AGATGC TAAAT GCGG CAG), 108r (CAGAAACCT TAAACCAT CTG) and V60LRI (5'-CATTG TCCAGTGTGC GCAGAC-3').

All reactions were performed using the Thermal Cycler 9600 (PerkinElmer). PCR products were separated in 2–3% agarose gels and were visualized in UV light. For samples in which a mutation was detected by the RFLP-PCR and allele-specific oligonucleotides (ASO) PCR analyses, a separate DNA sample was sequenced to confirm the presence of the mutation.

Statistics

To evaluate whether identified MC1R/CDKN2A alterations were associated with MM or breast cancer we compared the frequency of the detected variant in our patients to the control group from the general population, using logistic regression. This model is able to estimate not only the contribution of each particular polymorphism separately, but also possible interactions between them (e.g. multiplicative effects).

The differences in average age at diagnosis among the different genotypes were assessed using the t-test. Other features as clinical data or family aggregations were analysed using the common χ2 test. Possible deviation of the allele frequencies from those expected under Hardy–Weinberg equilibrium (HWE) was also assessed using the χ2 probability test.25

Linkage disequilibrium analysis was performed on the basis of the EM-Algorithm using LDlin1.0 software.27 The p-value of the likelihood ratio test was based on 1,000 iterations.

Haplotypic frequency analysis was performed using SNPAP v.1.3.1 (open-source software provided by David Clayton http://www.gene.cimr.cam.ac.uk/clayton/software/).

Results

Genomic sequencing of the familial cases 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. Since all the remaining variants (I155T, F196L, F300F, T314T, 3'C-UTR n965A/G) were very rare (each SNP present in single family only—less than 3% of familial cases) to reach statistical power we would have to examine thousands of cases and controls, numbers which can be obtained in multi-centre studies only; thus they were dropped from further analyses.

Haplotypic analysis in the control populations

There were no significant differences in the allele frequencies of the MC1R variants in the 2 control populations (Table 1). 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.

There were no significant differences in the 4 allele frequencies of the polymorphisms among males and females with a diagnosis of MM and among controls. There were no significant differences in the frequencies of the variants among newborns recruited throughout Poland (the regions of Szczecin/Gorzów/Zielona Góra, Białystok and Opole; data not shown).
MC1R COMMON VARIANTS AND CANCER RISK

TABLE I – FREQUENCIES OF THE EXAMINED MC1R VARIANTS IN CONTROLS AND SUBJECTS POPULATIONS

<table>
<thead>
<tr>
<th>SNP</th>
<th>Newborns</th>
<th>Melanoma-matched adults</th>
<th>Breast cancer-matched adults</th>
<th>Melanoma</th>
<th>Familial melanoma</th>
<th>Breast cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>V60L</td>
<td>111/793 (14)</td>
<td>60/421 (14.2)</td>
<td>82/511 (16)</td>
<td>85/467 (18.2)</td>
<td>8/40 (20)</td>
<td>92/511 (18)</td>
</tr>
<tr>
<td>R160C</td>
<td>118/778 (15.2)</td>
<td>68/421 (16.2)</td>
<td>81/511 (15.9)</td>
<td>89/462 (19.3)</td>
<td>8/40 (20)</td>
<td>83/511 (16.2)</td>
</tr>
<tr>
<td>R151C</td>
<td>70/793 (8.8)</td>
<td>38/421 (9)</td>
<td>46/511 (9)</td>
<td>73/474 (15.4)</td>
<td>7/40 (17.5)</td>
<td>44/511 (8.5)</td>
</tr>
<tr>
<td>R163Q</td>
<td>42/763 (5.5)</td>
<td>21/421 (5)</td>
<td>27/511 (5.3)</td>
<td>37/472 (7.8)</td>
<td>4/40 (10)</td>
<td>28/511 (5.5)</td>
</tr>
</tbody>
</table>

1Values in parentheses indicate percentage values.

TABLE II – LINKAGE DISEQUILIBRIUM

<table>
<thead>
<tr>
<th>SNP</th>
<th>V60L</th>
<th>R151C</th>
<th>R160C</th>
</tr>
</thead>
<tbody>
<tr>
<td>R151C</td>
<td>110.0051</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R160C</td>
<td>110.0080</td>
<td>0.9610.0051</td>
<td></td>
</tr>
<tr>
<td>R163Q</td>
<td>110.0025</td>
<td>110.0017</td>
<td>0.510.00072</td>
</tr>
</tbody>
</table>

1Format: D’1/2. The empirical p-value based on 1,000 permutations is lower than 1e − 12 for all combinations.

The expected allelotype distributions for all polymorphisms were in HWE irrespective of whether they were breast cancer cases, melanoma cases or controls.

Linkage disequilibrium

All 4 MC1R SNPs are in linkage disequilibrium with one another (p < 1e − 12). The empirical p-value is based on 1,000 permutations (Table II).

Haplotype frequency

We found no significant differences among haplotype composition among melanoma cases when compared with the newborn controls. The frequencies estimated for each haplotype are displayed in Table III.

Haplotype analysis in melanoma patient population

Since there were only few homozygous cases (e.g. 4 homozygous R160C MM carriers, 1 homozygous R163Q MM carrier, etc.) below we combined homozygous and heterozygous carriers. All 4 SNPs and their possible interactions where jointly analysed by the help of logistic regression.

We found a statistically significant association of the R151C variant (p = 0.000008; OR = 2.9, 95% CI 1.82–4.67), the V60L variant (p = 0.007; OR = 1.78; 95% CI 1.2–2.64), the R160C variant (p = 0.006; OR = 1.76; 95% CI 1.75–2.62) and the R163Q variant (p = 0.015; OR = 2.195% CI 1.1–3.97) with melanoma risk.

The analysis of possible interactions between those variants could not detect other effects than just linear ones (additively). However, the co-occurrence of 2 mutations had an effect on the mean age at diagnosis (see below).

The presence of any of those 4 common MC1R variants was significantly higher among patients than in the control population (χ² test; p = 0.0000004; OR 1.4).

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). Occurrence of breast cancer, however, was similar in both groups (Table IV). Analogously there was a slight increase in the prevalence of all MC1R variants among melanoma probands with first or second degree relative affected by melanoma (73/474; 15.4%) when compared to unselected cases (73/474; 15.4%; Table I).

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 = 0.0014; OR = 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 tumor type (Table V).

Haplotype analysis in breast cancer patient population

We found no association with the MC1R variants and breast cancer risk (Table I). There were no statistically significant differences in the prevalence of the MC1R variants among early and late onset cases (data not shown). None of the compound heterozygotes (2 or more MC1R changes, also MC1R alterations with the A148T CDKN2A variant) were significantly over-represented among cases (data not shown).

There was a tendency of increased frequency of melanoma occurrence among first degree relatives of carriers of any of the MC1R variants in comparison to non-carriers. Occurrence of breast cancer, however, was similar in both groups (Table VI).

Discussion

The results of this population-based study show herein that MC1R variants are associated with increased melanoma risk in the Polish population. Carrying any of the MC1R variant alleles was associated with almost 2-fold increase in risk of both sporadic and familial melanoma (p = 0.0000004). Interestingly our results are similar to those obtained in a study from northeastern Italy17 and in subjects of Celtic or German origin.2,8 Since we used larger groups of cases and controls, contrary to report of Landi et al.10
The strongest association was observed for the R151C variant. This is similar to the results of Van der Velden et al, who found that the MC1R variant R151C modified melanoma risk in Dutch melanoma-prone families.3 They concluded that the R151C variant is over-represented in patients with melanoma from families with the CDKN2A-Leiden mutation. The authors suggested that the R151C variant may be involved in melanoma tumorigenesis in a dual manner, both as a determinant of fair skin and as a component in an independent additional pathway, because the variant contributed to increased melanoma risk even after statistical correction for its effect on skin type.

Interestingly, in our study the R151C variant also showed the strongest association with CDKN2A (compound carriers of R151C(+)/A148T(+) genotype had a 4-fold increased MM risk). Since the R151C(+) /A148T(+) genotype is rare (present in 1.12% of melanomas and 0.27% of controls) we were unable to reach statistical power due to the limited number of cases. However, on the basis of reports indicating that both SNPs independently appear to be associated with an increased MM risk (R151C, A148T) this may be confirmed by larger multi-centre studies. Data obtained by the logistic regression model indicate that melanoma risk is increased 1.276-9.061 3.4

<p>| TABLE IV – OCCURRENCE OF MELANOMA AND BREAST CANCER AMONG I DEGREE RELATIVES OF MM PROBANTS |
|-------------------------------------------------|-------------------------------|----------------|-----------------|--------|</p>
<table>
<thead>
<tr>
<th>Feature</th>
<th>Carriers (+)</th>
<th>Non-carriers (-)</th>
<th>p-Value</th>
<th>95% CI</th>
<th>OR</th>
</tr>
</thead>
<tbody>
<tr>
<td>R160W1</td>
<td>4 (4.7%)3</td>
<td>17 (4.5%)3</td>
<td>n.s</td>
<td>1.269-8.966</td>
<td>3.4</td>
</tr>
<tr>
<td>BC occurrence</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V60L</td>
<td>5 (6.9%)</td>
<td>24 (6.7%)</td>
<td>n.s</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BC occurrence</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R163Q</td>
<td>5 (6.4%)</td>
<td>10 (2.8%)</td>
<td>n.s</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MM occurrence</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BC occurrence</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MM occurrence</td>
<td>17 (6.9%)</td>
<td>3 (2.0%)</td>
<td>0.03</td>
<td>1.05-12.60</td>
<td>3.6</td>
</tr>
<tr>
<td>BC occurrence</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

1. n = 85 for carriers (+) and 373 for non-carriers. 2. Number of malignant melanomas among I degree relatives. 3. Percentage of carriers with at least one I degree relative affected. 4. Percentage of non-carriers with at least one I degree relative affected. 5. Number of breast cancers among I degree relatives affected.

we were able to independently assess the association between each MC1R variant and melanoma risk. All the 4 common variants R151C, V60L, R160C and R163Q were significantly associated with MM risk. The haplotype analysis demonstrates that those variants do not co-occur in families with the CDKN2A-Leiden mutation. The authors suggested that the R151C variant may be involved in melanoma tumorigenesis in a dual manner, both as a determinant of fair skin and as a component in an independent additional pathway, because the variant contributed to increased melanoma risk even after statistical correction for its effect on skin type.
additively in compound MC1R(+) /CDKN2A(+) carriers suggesting that both genes do not share a common molecular pathway. Interestingly, we found an association between carrying any of the 4 variants and the increased risk of occurrence of melanoma among I degree relatives of the melanoma patients. However, most probably this risk is still too small to increase significantly the frequency of occurrence of familial aggregation of melanomas, due to low life-time risk in Central-European populations (1%).

The results of this study indicate that none of the common MC1R variants are associated with increased breast cancer risk. Thus MC1R cannot be added to the list of genes, which are believed to increase susceptibility both for common breast cancer and malignant melanoma, such as BRCA2, CDKN2A and XPD.

In conclusion, we found a modest association between 4 MC1R variants and melanoma risk in the Polish population (the highest OR for the R151C variant). The risk of disease seems to be increased additively if patients also harboured the CDKN2A common variant A148T: our results suggest a 4-fold increase of MM risk among compound carriers of MC1R-R151C(+) /CDKN2A-A148T(+) genotype. Additional studies are required to determine whether these particular changes can be associated with an increased risk of other malignancies at different sites of origin.

References


