MC1R gene variants and non melanoma skin cancer: a pooled-analysis from the M-SKIP project

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Abstract

Background: The Melanocortin-1-Receptor (MC1R) gene regulates human pigmentation and is highly polymorphic in populations of European origins. The aims of this study were to evaluate the association between MC1R variants and the risk of non-melanoma skin cancer (NMSC), and to investigate whether risk estimates differed by phenotypic characteristics.

Methods: Data on 3,527 NMSC cases and 9,391 controls were gathered through the M-SKIP Project, an international pooled-analysis on MC1R, skin cancer and phenotypic characteristics. We calculated Summary Odds Ratios (SOR) with random-effect models, and performed stratified analyses.

Results: Subjects carrying at least one MC1R variant had an increased risk of NMSC overall, basal cell carcinoma (BCC) and squamous cell carcinoma (SCC): SOR(95%CI) were 1.48(1.24-1.76), 1.39(1.15-1.69) and 1.61(1.35-1.91) respectively. All of the investigated variants showed positive associations with NMSC, with consistent significant results obtained for V60L, D84E, V92M, R151C, R160W, R163Q and D294H: SOR(95%CI) ranged from 1.42(1.19-1.70) for V60L to 2.66(1.06-6.65) for D84E variant. In stratified analysis, there was no consistent pattern of association between MC1R and NMSC by skin type, but we consistently observed higher SORs for subjects without red hair.

Conclusion: Our pooled-analysis highlighted a role of MC1R variants in NMSC development and suggested an effect modification by red hair colour phenotype.

Keywords: Pooled-analysis, pigmentation genes, genetic epidemiology, basal cell carcinoma, squamous cell carcinoma

Introduction

Non melanoma skin cancers (NMSC) are the most common malignancies in fair-skinned populations with a continuing increase in incidence during recent decades [Bath-Hextall et al, 2007, Flohil et al, 2011, Levi et al, 2001, Lomas et al, 2012]. According with the estimates of the American Cancer Society, more than two million NMSC are diagnosed annually in the US [American Cancer Society, 2012, Housman et al, 2003, Rogers et al, 2010]. In 1992, among US Medicare beneficiaries, NMSC ranked among the top five most costly cancers to treat [Housman et al, 2003]. Moreover, from 1992 to 2006 in the same population, there was a 77% increase in the total number of skin-cancer related procedures (around 94% NMSC [Rogers et al, 2010]). The vast majority of NMSC are basal cell carcinomas (BCC) and squamous cell carcinomas (SCC) with a BCC:SCC incidence ratio in immunocompetent patients of 4:1. BCC is the most common cancer in populations of European origin and accounts for 29% of all cancers [DePinho, 2000], although it is less likely to be lethal and rarely metastasizes. Previous studies identified solar UV irradiation, fair skin, red hair and freckles as the most relevant risk factors for NMSC development [International Agency for Research on Cancer, 2012, Rosso et al, 1996, Zanetti et al, 1996].

The Melanocortin-1-Receptor (MC1R) gene is involved in the genetics of human pigmentation. Binding of α-melanocyte-stimulating hormone (α-MSH) to MC1R stimulates the synthesis of melanin-activating adenylate cyclase enzyme, thereby elevating intracellular cyclic adenosine monophosphate (cAMP). Pigmentation is determined by regulation of the melanin proportion of photoprotective eumelanin and phaeomelanin, the latter being potentially mutagenic because it generates free radicals following UV exposure [Garcia-Borron et al, 2005].

MC1R is a highly polymorphic gene: more than 100 non-synonymous variants have been described to date [Garcia-Borron et al, 2005, Gerstenblith et al, 2007, Perez Oliva et al, 2009]. Functional analysis of some of these variants revealed partial loss of the receptor's ability to stimulate cAMP pathway leading to a quantitative shift of melanin synthesis from eumelanin to phaeomelanin [Duffy et al, 2004]. Phaeomelanin is associated with the "red hair colour" (RHC) phenotype, characterized by fair skin, red hair, freckles and sun sensitivity (solar lentigines and low tanning response) [Box et al, 1997]. Variant alleles of the following six single nucleotide polymorphisms rs1805006 (D84E), rs11547464 (R142H), rs1805007 (R151C), rs1110400 (I155T), rs1805008 (R160W) and rs1805009 (D294H) were defined as "R" alleles for their association with the RHC phenotype in population or familial association studies. The rs1805005 (V60L), rs2228479 (V92M) and rs885479 (R163Q) variants seem to have a lower association with RHC phenotype and have been designated as "r" alleles [Garcia-Borron et al, 2005]. Previous studies reported that the risk of NMSC is higher among carriers of MC1R variants [Bastiaens et al, 2001, Han et al, 2006, Kennedy et al, 2001, Scherer and Kumar, 2010, Smith et al, 1998]. However, it is not well known which variants are mostly associated with NMSC and whether the association completely depends on pigmentation characteristics.

The first aim of this study was to evaluate the association between specific and combined MC1R variants and the risk of NMSC through a large multicenter pooled-analysis of individual data from the Melanocortin-1 receptor gene, SKin cancer and Phenotypic characteristics (M-SKIP) project. The second aim was to evaluate whether risk estimates differed by phenotypic characteristics.

Materials and methods

Data for the present analyses were gathered through the M-SKIP project, which was previously described [Raimondi et al, 2012]. Briefly, we collected data from epidemiological studies on MC1R variants, sporadic cutaneous melanoma (CM), NMSC, and phenotypic characteristics associated with skin cancer from 33 investigators who agreed to participate to the M-SKIP project. Participant investigators sent their data along with a signed statement declaring that their original study was approved by an Ethics Committee and/or that study subjects provided a written consent to participate in the original study. We created a pooled database, including data on 8,301 CM cases, 3,542 NMSC cases and 15,589 controls.

For the present study, we identified in the M-SKIP database 8 independent case-control studies on NMSC [Andresen et al, 2013, Brudnik et al, 2009, Dwyer et al, 2004, Ferrucci et al, 2012, Kennedy et al, 2001, Liu et al, 2009, Nan et al, 2009, Scherer et al, 2008], that overall included data on 2,587 BCC cases, 788 SCC cases, 152 cases with both BCC and SCC, and 9,391 controls.

Statistical analysis

First, we compared population characteristics reported in publications of non-participant authors with those of studies included in our analysis, in order to assess the representativeness of our study population. Categorical and continuous variables were compared by the Chi-Square test and by the Wilcoxon two-sample test, respectively. Small-study effects was graphically represented by funnel plots and formally assessed by Egger's test. We verified the departure of frequencies of each MC1R variant from expectation under Hardy-Weinberg (HW) equilibrium by the Chi-Square test in controls for each included study.

We first pooled BCC and SCC together to evaluate the association between MC1R variants and NMSC risk overall, and then performed separate analyses to test the association of MC1R variants with BCC and SCC risk. For the first step we considered all 3,527 cases (2,587 BCC, 788 SCC and 152 with both), while for the second step we first considered 2,739 cases for BCC (2,587 BCC and 152 with both), and then 940 cases for SCC (788 SCC and 152 with both). For all the analyses controls are subjects free of any skin cancer.

We previously tested different inheritance models and found that the dominant model was the one with the lowest Akaike's Information Criterion for almost all the studies and variants, therefore we assumed this model of inheritance in the pooled-analyses [Pasquali et al, 2015]. For each study, we calculated the odds ratio (OR) with 95% Confidence Interval (CI) of MC1R variants by applying logistic regression to the data. Beyond MC1R, each model included, if available, the following covariates: age, sex, intermittent and chronic sun exposure, lifetime and childhood sunburns, and smoking status. Coding and standardization of the variables in the M-SKIP database has been described elsewhere [Raimondi et al, 2012]. For each study, we imputed missing data with multiple imputation models for variables with less than 20% of missing data, by using the iterative Markov chain Monte Carlo method, as previously described [Schafer, 1997]. We choose this method because of several datasets presented non-monotone missing data patterns and because it is robust to minor departures from the assumptions of multivariate normality [Schafer, 1997]. For each imputation procedure, five datasets were generated, which was considered an adequate number in multiple imputation [Rubin, 1996]. The results from the five logistic regression models applied to the imputed datasets were then combined for the inference with proc mianalyze (SAS software).

We performed the analysis using two different criteria to define the reference category for MC1R: the first one was applied to the 4 studies where MC1R was sequenced, and it used the wild-type (WT) subjects as a reference category for each variant; the second one was applied to the 4 studies where MC1R gene was not sequenced, and it used, for each study, subjects without any of the tested MC1R variants as a reference category for each variant. For this latter analysis, it should be noted that reference category includes both WT and carriers of any MC1R variant which was not specifically assessed in each original study (Table 1).

In all analyses, we took into account all the identified variants and calculated the OR for: 1) carrying at least one MC1R variant; 2) carrying just one MC1R variant; and 3) carrying ≥ 2 MC1R variants. Finally, a MC1R score was calculated, by summing across the MC1R alleles, giving a value of 1 to "r" and 2 to "R" variants. To calculate this score we considered both common and rare variants and classified them as previously suggested [Davies et al, 2012].

Following the two-stage analysis approach we pooled study-specific ORs using a random-effects model, implemented by the DerSimonian-Laird method. When there were more than one OR calculated in a single study (i.e. analysis by MC1R score), we took into account the correlation between the ORs by using the multivariate approach of van Houwelingn et al [van Houwelingen et al, 2002]. We evaluated homogeneity among study-specific estimates by the Q statistic and I^2, which represents the percentage of total variation across studies that is attributable to heterogeneity rather than to chance. We considered that statistically significant heterogeneity existed when the p-value was ≤ 0.10. When significant heterogeneity was revealed, we performed sensitivity analysis and meta-regression by year of publication of the study, geographic area where the study was carried out, MC1R genotyping methodology, deviation from HW equilibrium, type of controls and DNA source. In order to evaluate the robustness of the results,

we also compared the pooled-OR obtained using the M-SKIP dataset with the meta-OR calculated by pooling risk estimates reported in studies from not-participating investigators also using DerSimonian-Laird random-effects models.

We computed the Attributable Risk (AR) in the population for the presence of at least one MC1R variant and for the MC1R variants found to be statistically significantly associated with NMSC by using the Miettinen's formula: (OR-1/OR) x proportion of cases exposed, with the corresponding 95%CI.

Finally, we performed a stratified analysis in order to investigate whether the observed association between MC1R variants and NMSC varied according with different phenotypic characteristics. Phenotypic characteristics were taken into account only in stratified analysis and were not considered as confounders in previous analyses because they are likely in the pathway between MC1R and NMSC. Study-specific ORs were adjusted by age, sex, intermittent and chronic sun exposure, lifetime and childhood sunburns, and smoking status, where available. The hypothesis of homogeneity of ORs among strata was tested by meta-regression models with random-effects and restricted maximum likelihood estimates, after the calculation of strata-specific OR in each study. The correlation between the ORs calculated in the same studies was taken into account by using the approach of van Houwelingn et al [van Houwelingen et al, 2002].

The analysis was carried out using SAS (version 9.2) and STATA (version 11.2).

Results

Studies included in our pooled-analysis did not differ from studies from not-participating investigators according to publication period, study area, phenotype assessment, source of controls, genotyping methodology, mean age of cases and controls, sex distribution of cases and controls.

Table 1 summarizes the eight case-control studies included in the pooled analyses, four of which provided information on both BCC and SCC, three on BCC only, and one on SCC only. The studies were published between 2001 and 2013 and the majority of them were carried out in Europe (N=4/7 (57%) and N=3/5 (60%), for BCC and SCC subgroups, respectively). For the BCC analysis, hospital controls were recruited in 4 studies (57%) and population controls in 3 studies (43%), while for the SCC analysis, population controls were included in 3 studies (60%) and hospital controls in the remaining 2 studies (40%). All the studies included patients with histological confirmed diagnosis, except the Nurses Health Study [Nan et al, 2009] in which histological and self reported diagnosis were collected. For this latter study, however, the validity of self-reported diagnosis was reported to be 90% [Nan et al, 2009]. A complete sequencing analysis of the MC1R coding region was performed in 3 studies (43%) in BCC subgroup and in 2 studies (40%) in SCC subgroup. In general, cases were of a similar age, or slightly older than controls, and except for a study which only included women, the sex distribution was similar between cases and controls. Individual information on age and sex were available for each study, but information on other potential confounders varied between studies. Among the 8 included studies, no deviation from HW equilibrium was observed for the following MC1R variants: V60L, D84E, V92M, I155T, and R163Q. Deviation from HW equilibrium was observed in one study for R142H [Liu et al, 2009] and R151C [Dwyer et al, 2004], and in two studies for R160W

[Brudnik et al, 2009, Liu et al, 2009] and D294H [Andresen et al, 2013, Brudnik et al, 2009].

Further information on cases identification are presented in Supplementary Table S1.

Association between combined MC1R variants and NMSC

We found that subjects carrying any MC1R variant had a significantly increased risk of NMSC (Table 2) compared with subjects without any MC1R assessed variant. In more detail, carrying at least one MC1R variant increased the risk of NMSC overall, BCC and SCC: summary odds ratio (SORs) (95%CI) were respectively 1.48 (1.24-1.76), 1.39 (1.15-1.69) and 1.61 (1.35-1.91).

Carriers of two or more MC1R variants always presented higher SORs compared to subjects carrying one MC1R variant: SORs (95%CI) were 1.80 (1.49-2.17) for NMSC overall, 1.70 (1.36-2.12) for BCC and 2.10 (1.60-2.76) for SCC (Table 2).

We observed a significant linear trend for one point increase in MC1R score for NMSC overall, BCC and SCC: per-point SOR (95%CI) were 1.25 (1.14-1.36), p-value < 0.0001, 1.22 (1.11-1.34), p-value < 0.0001 and 1.28 (1.17-1.41), p-value < 0.0001, respectively (results not shown). We then restricted the analysis only to the studies that sequenced the MC1R gene. ORs for these studies were always higher than ORs obtained both on the whole group of studies and on the studies with MC1R not sequenced (p < 0.0001, results not shown) .

Association between single MC1R variants and NMSC

The nine most prevalent MC1R variants in the M-SKIP database were: V60L, D84E, V92M, R142H, R151C, I155T, R160W, R163Q, D294H. Table 3 and Supplementary Figure S1 present SORs for the association of each variant with NMSC in the whole group of 8 studies, using as reference group for each variant the subjects without any MC1R assessed variant. Table 3 also

presents results obtained restricting the analysis only to the studies that sequenced the MC1R gene. We found positive associations between all the investigated MC1R variants and NMSC, with SORs always higher than 1.00 for both the whole group of studies and the sequenced studies. Particularly, in the whole group of studies, a statistically significant association with NMSC overall was found for all variants except R142H and I155T. Furthermore, we observed a significant association with BCC for 6 MC1R variants: V60L, D84E, V92M, R151C, R160W and D294H, and a significant association with SCC for 6 variants: V60L, V92M, R151C, I155T, R160W and D294H. Significant heterogeneity was found for 5 variants in the NMSC pooled-analyses (D84E, R151C, I155T, R160W, R163Q) and for 5 variants in BCC (V60L, R151C, I155T, R160W, R163Q).

By meta-regression, we found that genotype methodology significantly affected the risk estimates for some analyzed variants, probably due to different reference categories. Restricting the analysis only to the studies that sequenced the MC1R gene, ORs were almost always higher than ORs obtained both on the whole group of studies and on the studies with MC1R not sequenced ($p < 0.0001$, results not shown). ORs significance was consistent for all MC1R variants with the only exception of the I155T that reached statistically significance for NMSC overall and BCC, while lost statistical power to confirm the association with SCC. Other possible sources of between-study heterogeneity, as publication year, study area, deviation from HW equilibrium, source of controls and source of DNA, seemed not to play a role to the observed heterogeneity. Otherwise, sensitivity analysis indicated that the heterogeneity may be attributable to single studies: when we excluded the studies that were outliers in the corresponding funnel plot, we obtained similar pooled-ORs than the original analysis, but with no more evidence of

heterogeneity among study-specific estimates (results not shown). Sensitivity analysis excluding studies not in HW equilibrium gave similar results.

Funnel plots for each MC1R variant are presented in Supplementary Figure S2 for the whole set of 8 studies. We found suggestion of small-study effects for R151C and R160W variants, with p-values of 0.021 and 0.006, respectively.

Figure 1 presents AR for MC1R variants significantly associated with NMSC in the previous analysis. The highest AR for both BCC and SCC was observed for R151C (7.3% and 11.1%, respectively), followed by R160W (7.0% and 11.2%, respectively).

Meta-ORs calculated for studies not included in the M-SKIP project were similar to those obtained from our pooled-analysis for all variants (results not shown).

Analysis stratified by phenotypic characteristics

Table 4 presents SORs for the association between NMSC and any MC1R variant stratified by skin type, hair colour and freckles. For this analysis, subjects without any MC1R assessed variant were the reference group for each variant. We consistently observed higher SORs for the association between MC1R and NMSC for subjects without red hair and without freckles. For hair colour, the difference between SOR of red haired and not red-haired subjects was statistically significant for SCC and borderline for NMSC overall (p=0.01 and 0.06, respectively), indicating that MC1R is more important for subjects with darker hair colour than with red hair. Similarly, although not significant, subjects without freckles have greater MC1R associated risk than subjects with freckles. Stratified analyses on the 4 studies that sequenced the MC1R gene were not feasible due to the limited sample size in each strata-specific analysis.

Discussion

We found a statistically increased risk of NMSC for carriers of at least one MC1R variant, with slightly higher SOR observed for SCC than for BCC. Although these two kinds of tumours share many similarities, they present rather different incidence rates and etiological factors. It has been suggested that neoplastic transformation of epithelial cells requires significantly less UV for BCC than for SCC [Rosso et al, 1996]. Cumulative exposure to sunlight was indeed found to be the main risk factor for SCC, while intermittent sun exposure plays a major role in BCC development [International Agency for Research on Cancer, 2012].

We consistently observed significant MC1R-associated NMSC risk only for subjects without red hair, while in subjects with red hair MC1R seemed not to have effect in addition to phenotype. Previous studies [Andresen et al, 2013, Bastiaens et al, 2001, Ferrucci et al, 2012, Liboutet et al, 2006, Scherer et al, 2008] also suggested that MC1R variants had an independent role in NMSC by phenotypic characteristics, and a similar finding has been observed for melanoma development [Pasquali et al, 2015]. MC1R may therefore contribute to skin carcinogenesis through other mechanisms than pigmentation. MC1R signaling has been implicated in a number of key biological pathways involved in cell cycle control [April and Barsh, 2007], apoptosis [Hauser et al, 2006], and activation of DNA repair mechanisms and antioxidant defenses [Bohm et al, 2005, Kadekaro et al, 2010, Kadekaro et al, 2012, Maresca et al, 2010]. In addition, stimulation of MC1R also activates the MAPK pathway and regulates target genes involved in inflammation through the NF-Kb pathway [Wikberg et al, 2000]. Finally, α-MSH affects proliferation and differentiation of both melanocytes and keratinocytes [Slominski et al, 1991]. Concerning individual variants, all of them showed a positive association with NMSC, with a consistent statistically significant association with either NMSC overall, BCC and SCC observed

for V60L, V92M, R151C, R160W and D294H. Functional studies revealed that these variants resulted in inefficient or even absent activation of the cAMP pathway downstream. Specifically, V60L, R151C and R160W variants reduced cell surface expression with a corresponding impairment in cAMP activation, while the loss-of-function phenotype of the D294H variant is probably due to inability to properly undergo the agonist-induced transition to the active state and/or to impaired coupling to the Gs protein [Beaumont et al, 2007, Herraiz et al, 2012, Schioth et al, 1999]. Only a marginal effect of the V92M substitution on cell surface expression or ability to activate the cAMP and ERK cascades has been reported [Beaumont et al, 2007, Herraiz et al, 2012].

The R163Q variant reached statistical significance only for the analysis of NMSC overall, while the D84E variant was associated with both NMSC overall and BCC. Furthermore, the I155T variant was associated only with SCC, although after restricting analysis to sequenced studies there was a suggestive association of I155T with BCC and NMSC overall. For D84E and I155T, receptor impairment in cAMP coupling is largely accounted by reduced cell surface expression [Beaumont et al, 2007, Sanchez-Laorden et al, 2007, Sanchez-Laorden et al, 2009]. It is not clear whether our results are attributable to a specific role of the above-mentioned variants in the pathogenesis of each tumour type. This would need to be further investigated in functional studies focused on the carcinogenic mechanisms leading to BCC and SCC, respectively.

Finally, the R142 variant did not show a statistically significant association with NMSC, probably due to its low allele frequency (<1%) and, consequently, limited statistical power.

In previous studies, contradictory results were reported for the associations of the most common MC1R variants and NMSC [Andresen et al, 2013, Bastiaens et al, 2001, Dwyer et al, 2004, Han et al, 2006, Jones et al, 1999, Liboutet et al, 2006, Scherer et al, 2008, Smith et al, 1998],

probably due to small sample size of single studies, especially for variants with relatively low allele frequencies.

To the best of our knowledge, our study has for the first time put together and meta-analyzed results from different studies on MC1R and NMSC, thus providing powerful estimates of the association between single and combined MC1R variants and NMSC risk in populations living in different geographical areas. Moreover, the availability of individual data from each study allowed a stratified analysis by phenotypic characteristics to be performed, and thus the independent contribution of MC1R variants on NMSC risk to be assessed. We were also able to provide both separate risk estimates for BCC and SCC and a combined risk estimate for NMSC overall. A further strength is that we took into account in our centralized statistical analysis all the available confounders, with a homogeneous plan of analysis and homogeneous definition of co-variables. Our results might have an important impact on public health since MC1R variants may be considered, along with other epidemiological risk factors, in both primary and secondary prevention strategies for NMSC, the most common neoplasm in populations of European origin. The improved identification of at risk subjects might enable public-health messages and early diagnostic procedures to be targeted to the population at risk.

One limitation of our study is that MC1R gene sequencing was completed in only 4 out of the 8 studies included in our pooled-analysis, and thus we were able to compare carriers of MC1R variants with wild type subjects only in a small subset of studies. As was previously pointed out [Pasquali et al, 2015, Williams et al, 2011], the inclusion of some MC1R variants in the reference category for the analyses would lead to underestimate the true risk of disease, because MC1R variants are very common among populations of European origins (66-67% of our study population had at least one variant). We sought to overcome this problem in our analysis by

excluding from the reference category all the MC1R variants that were specifically assessed in each study. Since the nine most common variants were examined in the majority of studies, the reference category would mainly include wild type and rare variants that were observed in around 4% of the study subjects in the M-SKIP dataset. We performed separate stratified analyses on subjects with and without red hair, skin type I/II and freckles, but unfortunately we could not compare subjects with and without any of these at risk phenotypic characteristics because they were collected jointly only in two studies [Ferrucci et al, 2012, Kennedy et al, 2001]. Lack of availability of information on other genes in most studies prevented the analysis of possible gene-gene interactions. Other genes have been indeed involved in NMSC development and include pigmentation genes like ASIP, TYR, TYRP1 OCA2, SLC, POMC and IRF4 [Nan et al, 2009, Scherer and Kumar, 2010], and, for BCC, the inactivating mutations in the PTCH gene [Liboutet et al, 2006]. Since we carried out a retrospective pooled-analysis, we did not perform centralized sequencing. However, previous studies [Davies et al, 2012, Harland et al, 2008] reported excellent concordance in sequencing data from different centres. Finally, differences in the assessment of sun exposure did not allow us to use this variable in stratified analysis, although it was possible to take it into account for the adjustment for confounders.

In conclusion, our pooled-analysis provided evidence for a role of all the most common variants in NMSC development, with consistent significant association with NMSC overall found for the MC1R variants V60L, D84E, V92M, R151C, R160W, R163Q and D294H. Since the contribution of MC1R variants in addition to phenotype in NMSC risk was mainly observed in subjects with no red-hair or no freckles, prevention strategies involving avoidance of indoor and outdoor ultraviolet radiation should not only be recommended for fair skin phenotypes, and MC1R assessment may be tailored to darker-pigmented subjects.

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Titles and legends to figures

Figure 1: Attributable Risks [a] **with 95% Confidence Intervals in the population for Non Melanoma Skin Cancer according to different MC1R variants** [b] **(percentages).**

Black bars represent BCC, white bars represent SCC.

[a] Miettinen's formula (OR-1/OR x proportion cases exposed).

[b] Only variants significantly associated with BCC and/or SCC are represented.