ELSEVIER

Cancer Genetics ■ (2013) ■

Cancer Genetics

Melanoma risk associated with *MC1R* gene variants in Latvia and the functional analysis of rare variants

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To evaluate the association of melanocortin 1 receptor gene (*MC1R*) variants with melanoma risk in a Latvian population, the *MC1R* gene was sequenced in 200 melanoma patients and 200 control persons. A functional study of previously uncharacterized, rare *MC1R* variants was also performed. In total, 26 different *MC1R* variants, including two novel variants Val165Ile and Val188Ile, were detected. The highest risk of melanoma was associated with the Arg151Cys variant (odds ratio (OR) 4.47, 95% confidence interval (CI) 2.19−9.14, $P < 0.001$). A gene dosage effect was observed, with melanoma risk for carriers of two variants being twice (OR 3.98, 95% CI 2.15−7.38, $P < 0.001$) that of carriers of one variant (OR 1.98, 95% CI 1.26−3.11, $P = 0.003$). After stratification according to the pigmentation phenotype, the risk of melanoma remained in groups with otherwise protective phenotypes. Functional analyses of eight previously uncharacterized *MC1R* variants revealed that a subset of them is functionally relevant. Our results support the contribution of *MC1R* variants to a genetic predisposition to melanoma in Latvia.

Keywords Melanocortin 1 receptor gene, melanoma, rare *MC1R* variants, pigmentation, functionality of *MC1R* variants

Cutaneous melanoma has shown a steady increase in incidence in the white population in the preceding decades, and this trend is likely to continue (1). Melanoma arises through the stepwise transformation of melanocytes within the basal epidermal layer of the skin and involves numerous molecular, cellular, and morphological events (2). Environmental sun exposure and individual's pigmentation phenotype factors have been strongly associated with the risk of melanoma (3). Approximately 10% of melanoma cases occur in a familial setting (4). To date, two genes, cyclin-dependent kinase inhibitor 2A (*CDKN2A*, 9p21.3, OMIM 600160) (5) and cyclin-dependent kinase 4 (*CDK4*, 12q14.1, OMIM 123829) (6), have been associated with high penetrance melanoma susceptibility. However, mutations in these genes account for susceptibility in only 20−57% of melanoma families (7) and have a very low frequency in melanoma patients at a population-based level (8). A genome-wide association study approach highlights several low penetrance melanoma-susceptibility candidate genes, including the melanocortin 1 receptor gene (*MC1R*, 16q24.3, OMIM 155555) (9), which is one of the major human skin pigmentation regulators (10). *MC1R* encodes a seven transmembrane domain, G-protein−coupled receptor of 317 amino acids. The binding of the natural agonist α-melanocyte-stimulating hormone (α-MSH) to a functional MC1R protein activates the intracellular signal cascade that leads to the production of the photoprotective brown or black eumelanin (11).

The *MC1R* gene is highly polymorphic with more than 100 nonsynonymous variants identified so far (12). Four variants—Asp84Glu, Arg151Cys, Arg160Trp, and Asp294His—present strong associations with the so-called red hair color (RHC) phenotype and are designated as RHC or R variants (13). The Arg142His and Ile155Thr variants are also sometimes labeled as RHC variants due to their strong

Received September 27, 2012; received in revised form January 11, 2013; accepted January 22, 2013.

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2210-7762/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.cancergen.2013.01.002

association with the RHC phenotype in a familial setting (13,14). Other variants, particularly Val60Leu, Val92Met, and Arg163Gln, present weaker associations with the RHC phenotype and therefore are labeled as NRHC or r variants (13). Regarding *MC1R* variants and melanoma risk, a recently performed meta-analysis summarizes results from different populations, confirming a strong link between *MC1R* variants (especially variants associated with the RHC phenotype) and risk of melanoma (15). Furthermore, *MC1R* variants have been associated with an increased melanoma risk, beyond already known phenotypic risk markers of melanoma, with a higher risk associated with darker hair and skin (16).

Most common *MC1R* variants have also been investigated in relation to their effects on receptor functional activity. Results from these studies demonstrated that MC1R with RHC variants have, to different extents, reduced receptor functional activity in a cyclic adenosine monophosphate (cAMP) assay (17–20) and/or reduced cell surface expression (17,21). However, little is known about the functional activity of other less common MC1R variants.

This is the first study that investigates the prevalence and type of *MC1R* variants among melanoma patients and control persons in a Latvian population and, to our knowledge, in the Eastern European region. In addition, we performed functional analyses of previously uncharacterized rare *MC1R* variants that were detected in our study population.

Materials and methods

Study population

From 2007 to 2011, 200 melanoma patients with histopathologically confirmed cutaneous melanoma were recruited at the Riga Eastern Clinical University Hospital Latvian Oncological Center. The control group consisted of 200 unrelated healthy volunteers without history of melanoma recruited through the general practice of family doctors. All participants enrolled in the study completed a questionnaire about their demographic characteristics and history of personal and familial cancers. Information on skin type according to the Fitzpatrick classification scale (type I: always burns, never tans; type II: burns easily, tans minimally; type III: sometimes burns, slowly tans; type IV: burns minimally, always tans), natural hair color, eye color, degree of freckling, and number of moles were also recorded. Each participant received an explanation of the aims of the study, agreed to participate, and signed an informed consent form approved by the Central Medical Ethical Committee of Latvia.

MC1R sequencing

Genomic DNA was extracted from peripheral blood lymphocytes by a standard phenol–chloroform extraction method. The entire *MC1R* coding sequence was amplified using primers: F 5′-GCA GCA CCA TGA ACT AAG CA-3′, R 5′-CAG GGT CAC ACA GGA ACC A-3′ (Metabion International AG, Martinsried, Germany). Polymerase chain reactions (PCRs) were performed in a 25 μL reaction volume containing 25 ng of template DNA, 1% Taq buffer, 10% dimethyl sulfoxide (DMSO), 1.5 mM magnesium chloride, 0.24 mM dNTPs, 4 μM of each primer and 1.25 U Taq DNA Polymerase (Thermo Scientific Molecular Biology, Waltham, MA). Cycling conditions were as follows: an initial denaturation at 95°C for 10 minutes followed by 35 cycles of denaturation at 95°C for 50 seconds, annealing at 61°C for 30 seconds, extension at 72°C for 1 minute, and a final extension at 72°C for 7 minutes. Amplicons were subjected to direct sequencing with four overlapping primers: S1F 5′-AAC CTG CAC TCA CCC ATG TA-3′, S1R 5′-CTG CAG GTG CTC ACG TCA AT-3′, S2F 5′-TCG TCT TCA GCA CGC TCT TC-3′, and S2R 5′-TTT AAG GCC AAA GCC CTG GT-3′. The sequencing reaction was performed using the ABI PRISM BigDye Terminator cycle sequencing kit (Applied Biosystems, Carlsbad, CA) and analyzed on an ABI PRISM 3100 Genetic analyzer (Applied Biosystems). Sequencing analysis was performed and confirmed manually using the Vector NTI (Life Technologies, Carlsbad, Ca).

Construction of *MC1R* variant clones

Variants of the *MC1R* gene with a frequency of less than 1% were analyzed for their effects on receptor functional activity. Variant Asp84Glu was excluded from these analyses as it is one of the RHC variants previously shown to impair receptor function (17,19). Similarly, the analyses excluded variant Tyr152X, which led to a predicted truncated inactivated protein, and variant Asp184His, due to its close position to the variant Val188Ile (assuming that their effects might be similar). For the remaining eight variants—Phe45Leu, Ser83Leu, Gly89Arg, Thr95Met, Asp121Glu, Val165Ile, Val188Ile, and Arg213Trp—expression constructs were generated. In addition, Arg151Cys was included in the study as a control variant since it has previously been shown to have reduced cell surface expression and functional activity in the cAMP assay (17,20,21). All expression constructs were prepared based on the vector pcDNA3.1+, which contains a human consensus *MC1R* sequence (Missouri S&T cDNA Resource Center, Rolla, MO). Polymorphic *MC1R* constructs were obtained by site-directed mutagenesis with overlap extension (22) using iProof High-Fidelity DNA Polymerase (Bio-Rad, Hercules, CA) and primers containing polymorphic mismatch or restriction sites (Supplementary Table 1). For the cAMP assay, amplified PCR fragments were subcloned into the pcDNA5/FRT (Life Technologies) between the NheI and XhoI sites. For the microscopy studies, *MC1R* variants without the stop codon were subcloned into the EcoRI and HindIII sites of the pGFP^2-N3 vector (PerkinElmer, Waltham, MA). For the microscopy, an expression construct for the melanocortin 2 receptor (MC2R) was made as an additional negative control that is not naturally transported to the baby hamster kidney (BHK) cell membrane due to the absence of the MC2R accessory protein MRAP (23), as previously described (24). Sequences of all obtained constructs were verified by plasmid sequencing using the ABI PRISM BigDye Terminator Cycle Sequencing Kit and ABI PRISM 3100 genetic analyzer (Applied Biosystems).

Cell culture and transfection

BHK cells obtained from the American Type Culture Collection, Manassas, VA, were grown at 37°C with 5% carbon

dioxide and maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% (v/v) fetal calf serum (FCS) and a penicillin–streptomycin mix (Sigma-Aldrich). When the cells reached 70–90% confluence, DNA constructs were transfected into the cells using TurboFect Transfection Reagent (Thermo Scientific Molecular Biology) according to the manufacturer's instructions. The cells were harvested and assayed 24 hours or 48 hours after transfection.

Confocal laser scanning microscopy

Cells were fixed in 4% paraformaldehyde (Sigma-Aldrich) for 10 minutes. Cell membrane and nuclei were stained with Alexa Fluor633 labeled wheat germ agglutinin (WGA) and 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Life Technologies), respectively. Cells were examined using Leica TCS SP2 confocal microscope (Leica Microsystems, Wetzlar, Germany), as previously described by Fridmanis et al. (24). Briefly, three images were obtained from each independent transfection reaction, and for each construct two transfection reactions were performed, therefore a total of six images were obtained for each construct. The efficiency of green fluorescent protein (GFP)–tagged receptor transport to the cell membrane was measured calculating the GFP/WGA fluorescence intensity ratio at multiple points on the cell membrane. Using the tool implemented in the Leica Confocal Software (Las AF version 2.6.0), 10 arbitrarily selected linear regions of interest (ROI) were drawn across each image, resulting in 20 points of intersection with the cell membrane and giving at least 120 points for analysis for each construct. To avoid ROI selection bias, ROIs were initially selected on the WGA image, and the respective GFP fluorescence intensity from the same ROI was obtained afterward.

cAMP assay

Transiently transfected BHK cells were distributed into a 384-well plate (approximately 1×10^4 cells/well) and stimulated with synthetic MC1R agonist NDP-MSH (PolyPeptide Group, Hillerød, Denmark) at different concentrations ranging from 10^{-12} to 10^{-6} M diluted in 1X phosphate-buffered saline, 1% bovine serum albumin, and 0.5 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich) for 30 minutes at 37°C. The intracellular cAMP level was measured with a LANCE cAMP kit (PerkinElmer) following the manufacturer's instructions and using a Victor3V multilabel reader (PerkinElmer). All experiments were performed in duplicate and repeated three times. Data was analyzed using the GraphPad Prism version 5.00 for Windows (GraphPad Software, La Jolla, CA).

Statistical analysis

Initially, we evaluated the associations between melanoma and known melanoma risk factors such as pigmentation characteristics using the Fisher exact test. The odds ratios (ORs) and the 95% confidence intervals (CIs) were calculated as well. The difference in mean age between groups was evaluated with an unpaired *t* test. For each *MC1R* variant, deviation from Hardy–Weinberg equilibrium was tested in controls as implemented in PLINK version 1.07 (Shaun Purcell, http://pngu.mgh.harvard.edu/purcell/plink/) (25). In our statistical analyses, all carriers of synonymous variants were pooled with persons with the *MC1R* consensus sequence; this pool was then considered as the wild type group. The association between melanoma risk and each *MC1R* nonsynonymous variant was analyzed separately as well as by combining different variants using the Fisher exact test along with ORs and 95% CIs. In the combination

Table 1 Risk of melanoma associated with phenotypic characteristics in a Latvian population

	Melanoma patients N = 200	Control persons N = 200			
Characteristic	No. (%)	No. (%)	OR	95% CI	*P* value
Hair color					
Black/Brown	85 (42.5)	126 (63.0)	1	-	-
Red/Fair	115 (57.5)	74 (37.0)	2.30	1.54–3.44	<0.001
Skin type					
III/IV	109 (54.5)	167 (83.5)	1	-	-
I/II	91 (45.5)	33 (16.5)	4.23	2.65–6.73	<0.001
Eye color					
Brown/other	52 (26.0)	62 (31.0)	1	-	-
Blue/gray/green	148 (74.0)	138 (69.0)	1.28	0.83–1.98	0.319
Freckles[a]					
None/few	147 (73.5)	167 (83.5)	1	-	-
Some/many	50 (25.0)	33 (16.5)	1.72	1.05–2.82	0.036
NA	3 (1.5)	0 (0)			
Moles[b]					
None/few	77 (38.5)	109 (54.5)	1	-	-
Some/many	117 (58.5)	91 (45.5)	1.82	1.22–2.72	0.004
NA	6 (3.0)	0 (0)			

Abbreviation: NA, not applicable.

[a] "None," no freckles at all; "Few," only a small number of freckles; "Some," a considerable number of freckles; "Many," a very high number of freckles on the face at the end of the summer.

[b] "None," no moles at all; "Few," only a small number of moles; "Some," a considerable number of moles; "Many, " a very high number of moles all across the body regardless of the mole size.

approach, we focused on the following *MC1R* variables: carriers of any *MC1R* variant, carriers of one or multiple (≥2) *MC1R* variants, carriers of one or more RHC variant (and no NRHC variant), carriers of one or more NRHC variant (and no RHC variant), and carriers of one or more RHC and one or more NRHC variants compared with the wild type group. RHC variants included those variants that have been previously shown to cause a significant impairment of receptor function and are strongly associated with red hair and fair skin, that is, the Asp84Glu, Arg151Cys, and Arg160Trp variants (26) and the Ser83Leu, Gly89Arg, Asp121Glu, and Arg213Trp variants that caused an impairment of receptor function similar to that of Arg151Cys in this study. All remaining nonsynonymous variants were considered NRHC variants. In addition, we explored the association between pigmentation characteristics and the presence of *MC1R* variants as well as the association between melanoma and RHC and NRHC variants after stratification according to the pigmentation characteristics. All *P* values cited are two-sided, and a *P* value less than 0.05 is regarded as statistically significant. All statistical analyses were performed using GraphPad Prism software version 5.00 for Windows.

To predict the functional impact of the *MC1R* variants on the receptor function, in silico analysis was performed using the software tool PolyPhen-2 (27). Statistical analyses of the confocal microscopy data were performed as described by Fridmanis et al. (24). A nonparametric Kruskal–Wallis test was first applied for uniformity analysis of the GFP/WGA fluorescence ratios per each construct ($\alpha = 0.05$). If statistically significant differences between medians were reported, Dunn's multiple comparison test ($\alpha = 0.05$) was used to determine which of the data sets were different. Data sets that significantly differed from more than two other data sets were replaced with data acquired from independent repeated experiments. Further, data from different constructs were analyzed by comparing median values and interquartile ranges using the Kruskal–Wallis test ($\alpha = 0.01$) followed by Dunn's multiple comparison test. Modules of differences in rank sums acquired by Dunn's test were arranged in a matrix

Table 2 *MC1R* variants identified in melanoma patients and control persons and each variant's individual association with melanoma risk[a]

		Melanoma patients N = 200	Control persons N = 200			
Nucleotide Change	Amino acid change	No. (%)	No. (%)	OR	95% CI	*P* value
Consensus	None	41 (20.5)	75 (34.1)	-	-	-
Synonymous						
g.399C>T	Cys133Cys	1 (0.5)	0 (0.0)	-	-	-
g.453C>G	Arg151Arg	1 (0.5)	0 (0.0)	-	-	-
g.498G>A	Ala166Ala	1 (0.5)	0 (0.0)	-	-	-
g.699G>A	Gln233Gln	2 (1.0)	2 (1.0)	-	-	-
g.819C>T	Cys273Cys	0 (0.0)	1 (0.5)	-	-	-
g.942A>G	Thr314Thr	64 (32.0)	53 (26.5)	-	-	-
g.948C>T	Ser316Ser	7 (1.8)	7 (1.8)	-	-	-
Nonsynonymous						
Wt[b]	None	47 (23.5)	84 (42.0)	1	-	-
g.133T>C	Phe45Leu	0 (0.0)	1 (0.5)	-	-	-
g.178G>T	Val60Leu	23 (11.5)	12 (6.0)	3.43	1.56–7.50	0.002
g.248C>T	Ser83Leu	2 (1.0)	0 (0)	-	-	-
g.252C>A	Asp84Glu	1 (0.5)	0 (0)	-	-	-
g.265G>C	Gly89Arg	0 (0)	1 (0.5)	-	-	-
g.274G>A	Val92Met	51 (25.5)	34 (17.0)	2.68	1.53–4.70	<0.001
g.284C>T	Thr95Met	1 (0.5)	0 (0)	-	-	-
g.363C>G	Asp121Glu	1 (0.5)	1 (0.5)	1.79	0.11–29.26	1.000
g.425G>A	Arg142His	8 (4.0)	8 (4.0)	1.79	0.63–5.07	0.286
g.451C>T	Arg151Cys	35 (17.5)	14 (7.0)	4.47	2.19–9.14	<0.001
g.456C>A	Tyr152X	1 (0.5)	0 (0)	-	-	-
g.464T>C	Ile155Thr	13 (6.5)	14 (7.0)	1.66	0.72–3.83	0.278
g.478C>T	Arg160Trp	49 (24.5)	35 (17.5)	2.50	1.43–4.39	0.002
g.488G>A	Arg163Gln	16 (8.0)	15 (7.5)	1.91	0.87–4.20	0.151
g.493G>A	Val165Ile	0 (0)	1 (0.5)	-	-	-
g.550G>C	Asp184His	0 (0)	1 (0.5)	-	-	-
g.562G>A	Val188Ile	1 (0.5)	0 (0)	-	-	-
g.637C>T	Arg213Trp	0 (0)	1 (0.5)	-	-	-
Insertion						
g.496_497insG	-	0 (0.0)	1 (0.5)	-	-	-

Abbreviation: Wt, wild type.

[a] Heterozygotes and homozygotes for a particular variant are grouped together (there were six patients homozygous for Thr314Thr, four for Arg160Trp, and one for each Val60Leu, Val92Met, Arg151Cys, Arg163GGln, Cys133Cys, or Arg151Arg variant, as well as three control persons homozygous for Val92Met, three for Thr60Thr, two for Arg160Trp, and one for Arg151Cys).

[b] Wt includes persons with the consensus *MC1R* sequence and carriers of synonymous polymorphisms.

table. The matrix table was used then to cluster constructs by their differences in expression on the cell surface. Clustering was performed with the Euclidean distance method using the MultiExperiment Viewer software version 4.3 (TM4 Development, Boston, MA)(28).

Results

Clinical and pigmentation characteristics of the study population

Overall, 400 persons were included in the study: 200 melanoma patients and 200 control persons. Melanoma patients and the control population did not differ in terms of gender ($P = 0.150$). There were 136 females (68%) and 64 males (32%) in the patient group and 150 females (75%) and 50 males (25%) in the control group. However, a younger mean age was observed in the control group when compared to the melanoma group: 48.7 years (±16.5 y) and 52.6 years (±15.5 y), respectively ($P = 0.012$). The pigmentation characteristics of the study participants as well as the risk of melanoma associated with each pigmentation phenotype are presented in Table 1. In the studied cohort, the strongest melanoma risk factors were fair skin types (OR 4.23, 95% CI 2.65−6.73, $P < 0.001$) and red or fair hair color (OR 2.30, 95% CI 1.54−3.44, $P < 0.001$). Other pigmentation characteristics (e.g., increased numbers of freckles and moles) were lower predictors of melanoma risk. No association with melanoma risk was observed for light eye color (Table 1).

Frequencies of *MC1R* variants and their association with melanoma risk

Altogether, 26 different *MC1R* variants were detected: 18 corresponded to nonsynonymous amino acid substitutions, seven resulted in synonymous changes, and one was an insertion (Table 2). Two of these variants, that is, Val165Ile (g.493G>A) and Val188Ile (g.562G>A), were novel low frequency variants that have not been previously reported. No variant showed a significant departure from Hardy−Weinberg equilibrium.

The frequencies of *MC1R* variants detected in the studied population are listed in Table 2. When nonsynonymous *MC1R* variants were examined individually, four variants (Val60Leu, Val92Met, Arg151Cys, and Arg160Trp) were significantly more frequent in melanoma patients, with the strongest risk for melanoma associated with the Arg151Cys variant (OR 4.47, 95% CI 2.19−9.14, $P < 0.001$) (Table 2). No other variant was individually associated with melanoma risk.

The presence of any *MC1R* variant was associated with a significant increase in melanoma risk when compared with that of the wild-type sequence (OR 2.36, 95% CI 1.53−3.63, $P < 0.001$). A gene dosage effect on melanoma risk in carriers of multiple variants was observed, with the OR for one variant equal to 1.98 (95% CI 1.26−3.11, $P = 0.003$) and the OR for 2 or more variants being twice as high (OR 3.98, 95% CI 2.15−7.38, $P < 0.001$) (Table 3). When both the number and type of variants are considered, a statistically significant increase in melanoma risk was associated with the presence of at least one RHC variant (OR 2.63, 95% CI 1.51−4.58, $P < 0.001$) and at least one NRHC variant (OR 1.90, 95% CI 1.16−3.10, $P = 0.013$). Moreover, the presence of both RHC and NRHC variants together increases melanoma risk approximately 1.4 and approximately 1.9 times more (OR 3.69, 95% CI 1.81−7.53, $P < 0.001$) when compared with the presence of at least one RHC or one NRHC variant, respectively (Table 3).

Association of *MC1R* variants with pigmentation characteristics

The associations among *MC1R* variants and pigmentation characteristics were examined using the control group. The presence of *MC1R* variants was associated with skin type I−II (OR 3.94, 95% CI 1.55−10.05, $P = 0.003$), red or fair hair color (OR 2.52, 95% CI 1.37−4.67, $P = 0.003$), and the presence of freckles (OR 3.94, 95% CI 1.55−10.05, $P = 0.003$). Furthermore, *MC1R* variants were not associated with eye color ($P = 0.642$) or mole count ($P = 0.567$) (Supplementary Table 2).

Associations among *MC1R* variants and melanoma risk after stratification by pigmentation characteristics

Table 4 shows the significant persistence of melanoma risk according to the type of *MC1R* variant after stratification for

Table 3 Associations among number and type of *MC1R* variants and melanoma risk

	Melanoma patients N = 200	Control persons N = 200			
Number and type of *MC1R* variant	No. (%)	No. (%)	OR	95% CI	*P* value
Wt[a]	47 (23.5)	84 (42.0)	1	-	-
Any	153 (76.5)	116 (58.0)	2.36	1.53−3.63	<0.001
1	104 (52.0)	94 (47.0)	1.98	1.26−3.11	0.003
≥2	49 (24.5)	22 (11.0)	3.98	2.15−7.38	<0.001
≥1 RHC[b]	53 (26.5)	36 (18.0)	2.63	1.51−4.58	<0.001
≥1 NRHC[c]	69 (34.5)	65 (32.5)	1.90	1.16−3.10	0.013
≥1 RHC and ≥1 NRHC	31 (15.5)	15 (7.5)	3.69	1.81−7.53	<0.001

[a] Wt includes persons with the consensus *MC1R* sequence and carriers of synonymous polymorphisms.
[b] RHC variants (Asp84Glu, Arg151Cys, Arg160Trp plus Ser83Leu, Gly89Arg, Asp121Glu, and Arg213Trp from the present study, which showed receptor functional impairment similar to that of Arg151Cys).
[c] NRHC variants (all other nonsynonymous variants).

Table 4 Role of *MC1R* variants on melanoma risk after stratification for pigmentation characteristics

Pigmentation characteristics	MC1R variant	Melanoma patients N=200 No. (%)	Control persons N=200 No. (%)	OR	95% CI	P value
Hair color						
Red/fair	Wt[a]	24 (12.0)	21 (10.5)	1	-	-
	Any	91 (45.5)	53 (26.5)	1.50	0.76–2.96	0.294
	≥1 RHC[b]	38 (19.0)	17 (8.5)	1.96	0.86–4.44	0.147
	≥1 NRHC[c]	34 (17.0)	28 (14.0)	1.06	0.49–2.30	1.000
	≥1RHC and 1 NRHC	19 (9.5)	8 (4.0)	2.08	0.75–5.72	0.215
Black/brown	Wt	23 (11.5)	63 (31.5)	1	-	-
	Any	62 (31.0)	63 (31.5)	2.70	1.49–4.88	0.001
	≥1 RHC	15 (7.5)	19 (9.5)	2.16	0.94–4.95	0.082
	≥1 NRHC	35 (17.5)	37 (18.5)	2.59	1.33–5.04	0.005
	≥1RHC and 1 NRHC	12 (6.0)	7 (3.5)	4.70	1.65–13.39	0.006
Skin type						
I/II	Wt	21 (10.5)	6 (3.0)	1	-	-
	Any	70 (35.0)	27 (13.5)	0.74	0.27–2.04	0.631
	≥1 RHC	25 (12.5)	11 (5.5)	0.65	0.21–2.06	0.571
	≥1 NRHC	27 (13.5)	13 (6.5)	0.59	0.19–1.83	0.418
	≥1RHC and 1 NRHC	18 (9.0)	3 (1.5)	1.71	0.37–7.86	0.712
III/IV	Wt	26 (13.0)	78 (39.0)	1	-	-
	Any	83 (41.5)	89 (44.5)	2.80	1.64–4.78	<0.001
	≥1 RHC	28 (14.0)	25 (12.5)	3.36	1.67–6.76	<0.001
	≥1 NRHC	42 (21.0)	52 (26.0)	2.42	1.33–4.43	0.004
	≥1RHC and 1 NRHC	13 (6.5)	12 (6.0)	3.25	1.32–8.01	0.014
Eye color						
Blue/gray/green	Wt	35 (17.5)	56 (28.0)	1	-	-
	Any	113 (56.5)	82 (41.0)	2.21	1.33–3.67	0.002
	≥1 RHC	40 (20.0)	27 (13.5)	2.37	1.24–4.52	0.010
	≥1 NRHC	51 (25.5)	47 (23.5)	1.74	0.97–3.10	0.079
	≥1RHC and 1 NRHC	22 (11.0)	8 (4.0)	4.40	1.77–10.96	0.001
Brown/other	Wt	12 (6.0)	28 (14.0)	1	-	-
	Any	40 (20.0)	34 (17.0)	2.75	1.21–6.21	0.018
	≥1 RHC	13 (6.5)	9 (4.5)	3.37	1.14–9.99	0.033
	≥1 NRHC	18 (9.0)	18 (9.0)	2.33	0.91–5.98	0.101
	≥1RHC and 1 NRHC	9 (4.5)	7 (3.5)	3.00	0.91–9.93	0.125
Freckles[d]						
Some/many	Wt	8 (4.0)	6 (3.0)	1	-	-
	Any	42 (21.0)	27 (13.5)	1.17	0.36–3.74	1.000
	≥1 RHC	14 (7.0)	10 (5.0)	1.05	0.28–3.99	1.000
	≥1 NRHC	14 (7.0)	13 (6.5)	0.81	0.22–2.97	1.000
	≥1RHC and 1 NRHC	14 (7.0)	4 (2.0)	2.63	0.57–12.18	0.267
None/few	Wt	37 (18.5)	78 (39.0)	1	-	-
	Any	110 (55.0)	89 (44.5)	2.61	1.61–4.22	<0.001
	≥1 RHC	39 (19.5)	26 (13.0)	3.16	1.68–5.95	<0.001
	≥1 NRHC	54 (27.0)	52 (26.0)	2.19	1.27–3.78	0.006
	≥1RHC and 1 NRHC	17 (8.5)	11 (5.5)	3.26	1.39–7.65	0.008
	NA	3 (1.5)	0 (0.0)		-	-
Moles[e]						
Some/many	Wt	28 (14.0)	36 (18.0)	1	-	-
	Any	89 (44.5)	55 (27.5)	2.08	1.15–3.78	0.023
	≥1 RHC	29 (14.5)	18 (9.0)	2.07	0.96–4.47	0.084
	≥1 NRHC	42 (21.0)	32 (16.0)	1.69	0.86–3.31	0.172
	≥1RHC and 1 NRHC	18 (9.0)	5 (2.5)	4.63	1.53–14.01	0.007
None/few	Wt	18 (9.0)	48 (24.0)	1	-	-
	Any	59 (29.5)	61 (30.5)	2.58	1.35–4.94	0.005
	≥1 RHC	22 (11.0)	18 (9.0)	3.26	1.43–7.44	0.007
	≥1 NRHC	26 (13.0)	33 (16.5)	2.10	1.00–4.43	0.061
	≥1RHC and 1 NRHC	11 (5.5)	10 (5.0)	2.93	1.07–8.08	0.061
	NA	6 (3.0)	0 (0.0)	-	-	-

[a] Wt includes persons with consensus *MC1R* sequence and carriers of synonymous polymorphisms.

[b] RHC variants (Asp84Glu, Arg151Cys, and Arg160Trp, plus Ser83Leu, Gly89Arg, Asp121Glu, Arg213Trp from the present study, which showed receptor functional impairment similar to that of Arg151Cys).

[c] NRHC variants (all other nonsynonymous variants).

[d] "None," no freckles at all; "Few," only a small number of freckles; "Some," a considerable number of freckles; "Many," a very high number of freckles on the face at the end of the summer.

[e] "None," no moles at all; "Few," only a small number of moles; "Some," a considerable number of moles; "Many, " a very high number of moles all across the body regardless of the mole size.

the pigmentation characteristics. In the studied population, melanoma risk associated with *MC1R* variants appeared to increase significantly in the presence of protective phenotypes such as black or brown hair, skin type III or IV, and decreased amount of freckling. Regarding eye color, the melanoma risk associated with *MC1R* variants was similar in persons with light and dark eye color. A melanoma risk similar to that associated with *MC1R* variants was found in persons with a low or high number of moles (Table 4). The same result was observed when melanoma risk was evaluated according to number of *MC1R* variants (data not shown).

Associations among *MC1R* variants, age of melanoma onset, and tumor characteristics

A statistically significant association between the age of melanoma onset and *MC1R* genotype was found. The median age at diagnosis was lower for noncarriers of *MC1R* variants than for carriers: 49 years and 55 years, respectively ($P = 0.033$). With respect to the anatomic distribution of the tumor, we observed the highest *MC1R* variant frequencies in patients with melanomas on the trunk (36.0%), followed by extremities (29.5%), and head or neck (10%). However, no statistically significant difference was found among these groups ($P = 0.858$). Similarly, no statistically significant difference was found when both the number and type of variants were taken into account (data not shown). We did not identify any relationship between the presence of *MC1R* variants and the Breslow thickness of the tumor. The distribution of variants was similar in tumors below and above the median tumor thickness of 3.0 mm ($P = 0.701$). Similarly, no association was found between tumor ulceration and *MC1R* variants ($P = 0.694$).

Functional studies of rare *MC1R* variants

In our study, 11 *MC1R* variants had a frequency of less than 1% (Table 2). Of these, Asp84Glu has been previously shown to impair receptor functions (17,19), and Tyr152X led to a predicted truncated inactivated protein. Five variants (Phe45Leu, Ser83Leu, Gly89Arg, Thr95Met, and Asp121Glu) were predicted in silico to be possibly damaging for receptor functions, and four others (Val165Ile, Asp184His, Val188Ile, and Arg213Trp) were predicted to be benign. The functional properties of cloned *MC1R*-variant alleles were addressed using transiently transfected BHK cells. First, confocal scanning microscopy of GFP-tagged wild-type and variant MC1R proteins (Figure 1) revealed that cell surface expression was strong for wild type MC1R and Phe45Leu, Thr95Met, Val165Ile, and Val188Ile allelic variants, whereas the Asp121Glu and Arg213Trp allelic variants showed markedly reduced and Ser83Leu and Gly89Arg allelic variants showed almost no detectable cell surface expression. The MC1R Arg151Cys variant and MC2R wild type showed significantly reduced or almost no detectable cell surface expression. Quantification of GFP levels in the plasma membrane of the cells confirmed the microscopy results (Figure 2). Next, variant MC1R functions were assessed by the ability of the cloned variants to elevate intracellular cAMP level in response to the agonist NDP-MSH. Only one variant (Gly89Arg) was a complete loss-of-function (LOF) receptor (Figure 3). All other variants showed an increase in cAMP in response to agonist administration, however, each to a different extent. The Thr95Met, Val165Ile, and Val188Ile variants showed essentially similar levels of maximal response as that of the wild type (Figure 3), and similar half maximal effective concentration (EC_{50}) values, except for the Val165Ile variant, for which a slightly increased EC_{50} value was observed (Supplementary Table 3). The Ser83Leu, Asp121Glu, and Arg213Trp variants did show reduced levels of maximal response and slightly increased EC_{50} values in comparison with those of the wild type (Figure 3, Supplementary Table 3). Conversely, the Phe45Leu variant showed a markedly increased level of cAMP response compared with that of the other variants (Figure 3).

Discussion

Numerous studies have shown that *MC1R* is highly polymorphic in the white population (12). In our study, the frequency of *MC1R* variants in the control population (58%) was intermediate compared with the frequencies reported in North European and Mediterranean populations, which were highest in Britain/Ireland and lowest in Greece (29). Similarly, the influence of *MC1R* variants on genetic predisposition to melanoma has been investigated in many association studies (10,13,16,30–35). Most of these studies demonstrated a strong association between *MC1R* variants and the risk of melanoma development, with an OR varying between 2 and 7, depending on the number and type of variants present (15,26,30,33–35). A similar gene dosage effect was observed in the present study, increasing the melanoma risk by a factor of 2 when one variant was present, and by 4 when at least two variants were present (Table 3). Evidence from functional studies indicates that compound heterozygotes and homozygotes of specific MC1R variants have a reduced cAMP response to the α-MSH hormone compared with that of simple heterozygotes (36). Therefore, we postulate that carriers of multiple variants may have a deeply impaired receptor function, but additional studies are needed.

Separate analyses of individual *MC1R* variants showed that four variants were significantly associated with an increased risk of melanoma, namely, Val60Leu, Val92Met, Arg151Cys, and Arg160Trp, with the strongest association being for Arg151Cys (OR 4.47; 95% CI 2.19–9.14, $P < 0.001$). Variants Arg151Cys and Arg160Trp have been strongly linked to melanoma risk in many relevant studies and were associated with the highest risk for melanoma in two recent meta-analyses (15,26). Furthermore, the association of Arg151Cys and Arg160Trp variants with melanoma and their functional impairments of the MC1R protein are well documented (17,20,21). The role of Val60Leu in melanoma development is controversial, as the magnitude of melanoma risk it confers varies among studies. Reports from Australia (10), the Netherlands (31), and Germany (34) have shown a weak or no association with melanoma risk. In contrast, studies of French (33) and Greek (35) populations demonstrated findings similar to those of our study, with Val60Leu serving as a strong predictor of melanoma risk. Similarly, the results for variant Val92Met have been conflicting among various populations and two recently performed

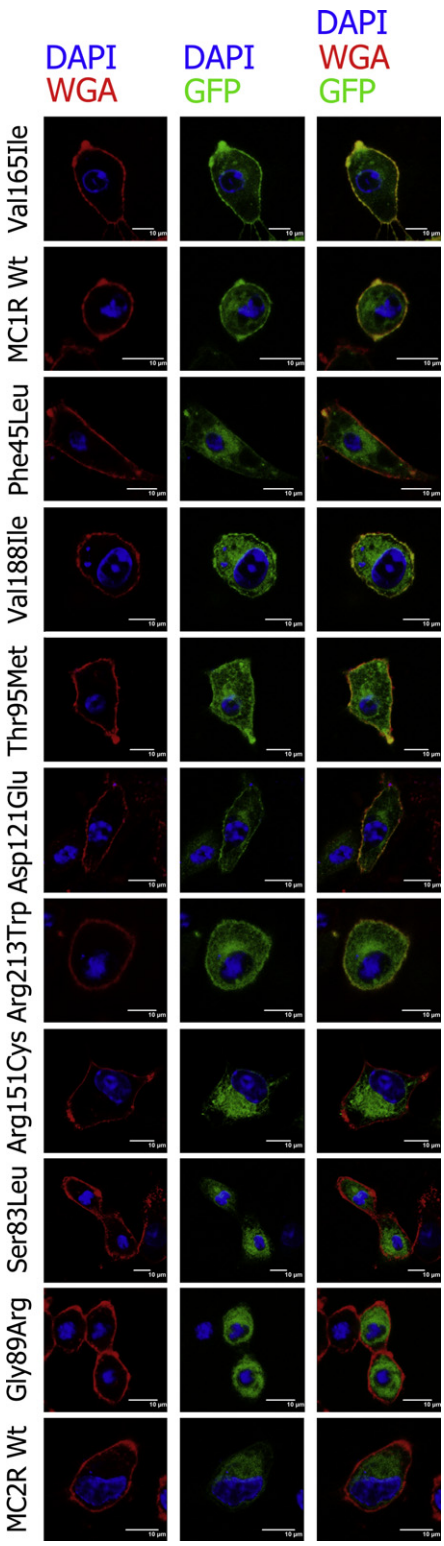

Figure 1 Confocal laser scanning microscopy images of GFP-tagged constructs with wild type MC1R and variant MC1R (Phe45Leu, Ser83Leu, Gly89Arg, Thr95Met, Asp121Glu, Arg151Cys, Val165Ile, Val188Ile, Arg213Trp) in BHK cells. Constructs are ranked from the highest (top) to the lowest (bottom) membrane expression. Wild type MC2R, which is known to be unexpressed on the BHK cell surface without an accessory protein, in this study was used as a negative control for receptor membrane export. The first column represents images of membrane staining with Alexa Fluor 633 conjugated WGA (red). The second column represents fluorescence images of the GFP tag (green). The third column represents a merge of images from cell membrane and GFP expression. Nuclei are always blue (DAPI).

meta-analyses (15,26). Two variants that were previously associated with an important melanoma risk (15,26), Asp84Glu and Asp294His, were detected in only one patient and not in controls or were not found in our population, respectively. Other variants that have been previously linked to melanoma risk, such as Arg142His and Ile155Thr, were not found to have a significant association in our cohort. This could be related to the small size of our affected group.

Several studies have noted that the association of *MC1R* variants with melanoma risk was stronger or limited to persons with protective cutaneous phenotypes, that is, persons with darker hair and darker skin (10,16,31,33). Here, we confirmed the persistent effect of *MC1R* variants on melanoma risk after stratification for the different pigmentation characteristics (Table 4), showing that *MC1R* variants and pigmentation are independent melanoma risk factors. Thus, our results suggest that the *MC1R* genotype provides information about melanoma risk beyond that of the cutaneous phenotype and that the combination of *MC1R* genotype and phenotype data might be important to the prediction of melanoma risk in persons with otherwise protective cutaneous phenotypes.

Previous work has shown that the presence of *MC1R* variants is associated with melanoma thickness, which is one of the main measures of tumor progression (32). However, similar to the study of the Greek population (35), our study found no such association, probably due to a higher median thickness of melanomas in our study. A lower percentage of thin tumors might prohibit us from finding a difference between *MC1R* variant carriers and noncarriers with respect to tumor thickness. Unlike other studies (32,35), our analysis found an association between *MC1R* variants and age of melanoma onset. Carriers of *MC1R* variants were older in our study than in other reports, and this difference cannot be readily explained. However, recently a protective effect of *MC1R* variants was observed on death from melanoma (37).

In the context of association studies, an important question is the classification of different *MC1R* variants, especially those whose frequencies are relatively low and for which it is difficult to evaluate their effect in association studies due to the lack of statistical power. Functional characterization of different *MC1R* variants could solve this problem. At present, however, the functional relevance is mainly determined for more frequently occurring RHC variants showing that these alleles encode for partial LOF receptors with diminished plasma membrane trafficking and ability to activate the cAMP pathway (17–20). However, the degree of residual signaling varies even between different RHC alleles, and it is not known whether other NRHC alleles maintain the same signaling capacity. This situation is further complicated by the fact that, at present, functional characterization is available for only a relatively small number of NRHC variants (12,38–41). In this study, we analyzed eight previously functionally uncharacterized rare *MC1R* variants that were found in a Latvian population. Two among them were novel (Val165Ile and Val188Ile). Four of these eight variants

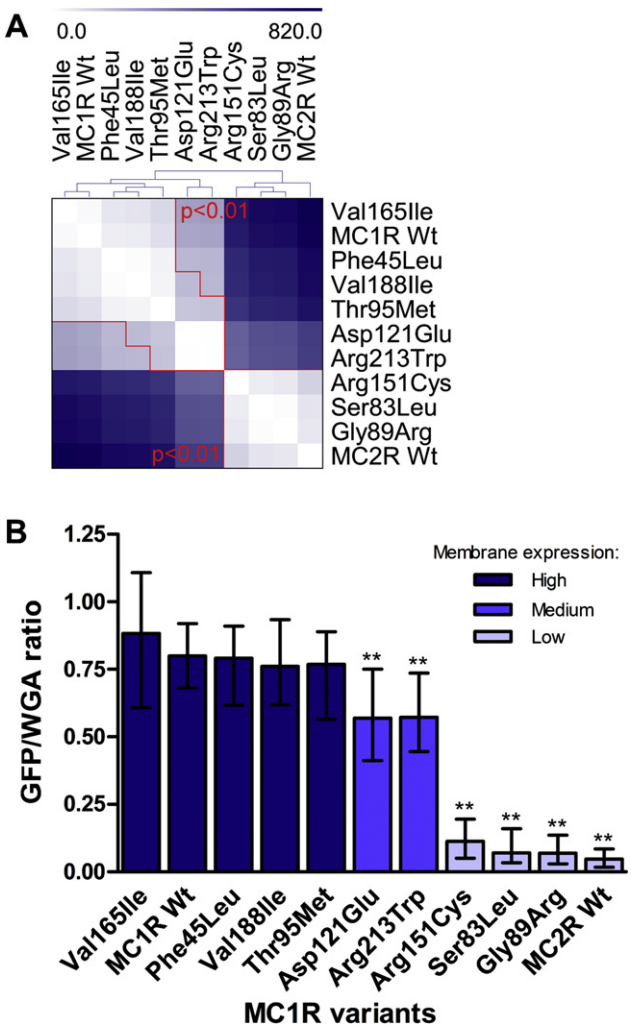

Figure 2 Quantitative results of membrane expression of MC1R variants. (A) A matrix table and MC1R-variant clustering tree for cell membrane export created using rank sums from Dunn's multiple comparison tests. The largest differences in rank sums are shown as dark blue boxes, white boxes correspond to the smallest differences, and color transitions from dark blue to white represent gradual decrease of differences. Red lines indicate borders between cells with significant difference in rank sums ($P < 0.01$) and cells without statistically significant differences in rank sums ($P > 0.01$). (B) A bar graph representing median values with interquartile ranges of GFP/WGA fluorescence intensity ratios at the cell membrane for all analyzed MC1R variants; $^{**}P < 0.01$ compared with wild type MC1R. The results are representative of at least two independent experiments.

(Phe45Leu, Ser83Leu, Gly89Arg, and Thr95Met) are located in the MC1R region that is involved in the formation of a ligand-binding pocket (42). One variant, Asp121Lys (we analyzed a variant in the same sequence position but with a different amino acid substitution, Asp121Glu), has been shown to be directly involved in ligand binding (43). In silico, all five variants participating in ligand binding were predicted to be damaging, whereas the others (Val165Ile, Val188Ile, and Arg213Trp), including both novel variants, were predicted to be benign. However, these predictions were not completely consistent with the results from in vitro analyses. Functional analyses of variants showed that Thr95Met and Val188Ile behaved as wild type variants in terms of both plasma membrane trafficking and signaling via the cAMP pathway. Variant Val165Ile had a cell surface density similar to that of the wild type and only slightly reduced signaling via the cAMP pathway. The other variants displayed varying degrees of LOF, indicating that LOF *MC1R* variants are more common than functionally silent variants, at least in the white population. The behavior of the Ser83Leu variant was reminiscent of that of the strong and frequent variant Arg151Cys, which has been shown to display decreased signaling via the cAMP pathway (20) and reduced cell surface export (21). The LOF of the variant Gly89Arg might also be caused by the reduction of cell surface expression with, accordingly, undetectable cAMP signaling. This phenotype is similar to other previously reported natural variants, such as Leu93Arg (41) and Arg162Pro (38,39). Similarly, the reduced cAMP signaling of variants Asp121Glu and Arg213Trp might be explained by the reduction in cell surface expression, as shown by confocal microscopy. Based on these observations, rare MC1R variants Ser83Leu, Gly89Arg, Asp121Glu, and Arg213Trp should be considered alleles whose residual signaling is comparable to that from RHC alleles. In contrast to all other MC1R variants, the variant Phe45Leu demonstrated a functional cAMP response higher than that of the wild type, with cell surface expression comparable with that of the wild type. The reason for such an increase in the cAMP level is not clear.

The exact mechanism underlying the influence of *MC1R* variants on the development of melanoma is not clear. However, a previous study has shown that MC1R variants, in comparison with the wild type receptor, might have an advantage in early melanoma development due to better proliferation rates and more effective binding of melanoma cells to the extracellular matrix (44). MC1R might also perform some immune-related function, because it is expressed on a variety of immune cells (45).

Our study has several limitations. First, the size of the association-study population was small and it did not have the power to detect an association for rare *MC1R* variants. For the same reason, the results from the subgroup analyses should be considered with caution. Although the sample size was not large even for the primary analysis, some of the subgroups were small with limited ability to detect associations. Multiple testing for the association analysis were not performed as a result of the sample size, which might have led to the overestimation of some *P* values. Second, the control participants enrolled in the study on a volunteer basis, which may have caused a selection bias. Another limitation was the self-reported pigmentation characteristics that, due to individual subjectivity, might be inaccurate. Regarding the in vitro analyses of the MC1R variants, our model system consisted of cells without melanocytic origin. Previous reports have shown that the same MC1R variant reduced cAMP levels to distinctive levels in different cell lines, though results from the different cell lines were not conflicting and the overall tendency remained the same (21). Therefore, we believe that our results for the BHK cell system are comparable with data from similar studies. In addition, our results on widely examined Arg151Cys are overall consistent with results from previous research (20,21).

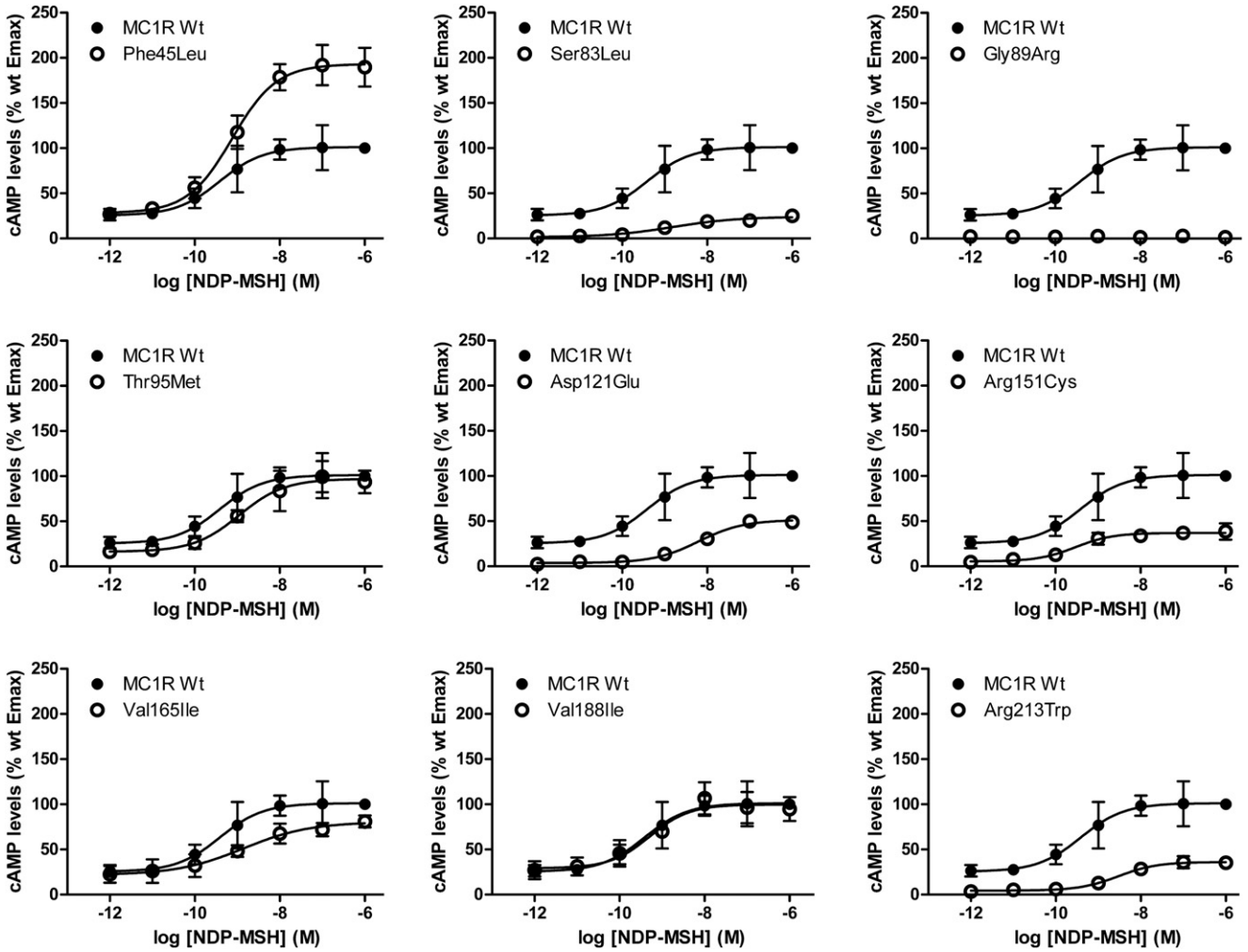

Figure 3 Functional coupling of variant MC1R to the cAMP production. Dose–response curves for induced cAMP production in BHK cells expressing the wild type or variant receptor in response to increasing concentrations of the agonist NDP-MSH. Results are the mean ± standard deviation (SD) of at least three independent experiments performed in duplicate.

In conclusion, for the first time we have determined the occurrence of *MC1R* variants in a Latvian population and evaluated their possible association with melanoma risk. Additionally, we have functionally investigated eight previously uncharacterized *MC1R* variants and demonstrated that a significant subset of these variants are functionally relevant. Our results increase the overall knowledge of the functional properties of MC1R and may help to classify *MC1R* variants more accurately in future studies.

Acknowledgment

This work was supported by the grant of European Economic Area (EEA) Nr. EEZ09AP-38/08 and the European Regional Development Fund (ERDF) project No. 2010/0233/2DP/2.1.1.1.0/10/APIA/VIAA/076. We acknowledge Genome Database of Latvian Population, Latvian Biomedical Research and Study Center for providing DNA samples. We thank Davids Fridmanis for assistance with the statistical analysis, Alexander Rivosh for assistance with the pictures, and Anna Azarjana for language editing.

Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.cancergen.2013.01.002.

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