RESEARCH NOTE

CDKN2A and *MC1R* variants found in Cypriot patients diagnosed with cutaneous melanoma

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Introduction

The prevalence of genetic variants associated to cutaneous melanoma (CM)has never been determined within Cypriot melanomas. This study, evaluates the frequency of variants in cyclin-dependent kinase inhibitor 2A (*CDKN2A*) and melanocortin-1 receptor (*MC1R),* in 32 patients diagnosed with CM. Other characteristics and risk factors were also assessed. *CDKN2A*p.Ala148Thr was detected in3 out of 32 patients, while the control group revealed no variationswithin *CDKN2A*. *MC1R*screening in 32 patients revealed the following variations: p.Val60Leu in 11 patients, p.Arg142His in 4 patients, p.Thr314Thr in 1 patient, p.Arg160Trp in 1 patient, p.Val92Met/p.Thr314Thr in1 patient and p.Val92Met/p.Arg142His/p.Thr314Thr in 1 patient. The control group revealed only p.Val60Leu (in 10 out of 45 individuals), frequentlyfound in general populations. Two unrelated patients carried*CDKN2A* p.Ala148Thr in combination with *MC1R* p.Arg142His, suggesting digenic inheritancethat may give evidence of different gene variants acting synergistically to contribute to CM development.This study confirms the presence of *CDKN2A* and *MC1R* variants among Cypriot melanomas and supports existing evidence of a role for these variants in susceptibility to melanoma.

CM is a complex disease that involves genetic and other risk factors. Exposure to sunlight and other phenotypic factors such as the occurrence of atypical melanocytic nevi, skin and hair fairness, blue eyes, and freckling seem to also play an important role (MacKie *et al.* 2009).The most important known high-risk melanoma susceptibility locus is *CDKN2A*, located on 9p21(Hill *et al.* 2013). This gene encodes two proteins: p16INK4a, involved in the regulation of cell growth, and also p14ARF which activates tumor suppressor p53(Sharpless and Chin 2003). Germline mutations/deletions in *CDKN2A*(p16INK4a) have been associated with familial melanoma(Hussussian *et al.* 1994).

Furthermore, *CDKN2A* mutations penetrance seems to be increased by the co-inheritance of *MCIR* variants (Demenais *et al.* 2010).*MC1R*, found to be linked to characteristics such as red hair, fair skin, and poor tanning ability, is a gene that codes for a seven-pass transmembrane G-protein-coupled receptor expressed on melanocyte cell surface. This receptor regulates melanin synthesis through binding to the alpha-melanocyte-stimulating hormone (α-MSH) causing a switch from red/yellow pheomelanin to brown/black eumelanin(Rees 2000). Binding of α-MSH to MC1R stimulates cAMP production, a major pathway leading to melanogenesis.Variations in *MC1R* seem to increase risk for melanoma secondary to intensified UV-mediated DNA damage due to absent photoprotectiveeumelanin or through accumulation of pheomelanin(Rees 2000).

Cyprus is reported to have a low incidence of melanoma among European countries with an estimated age-standardized incidence rate of 4.4 cases per 100,000 inhabitants(Ferlay *et al.* 2013). Although the genetic variability linked to melanoma has been extensively studied in numerous populations of various geographical areas, the incidence of genetic variants has not been determined within the Cypriot population.In order to assess the presence of an underlying genetic susceptibility to the development of CM, we screened 32 Cypriot patients diagnosed with CMto examine the occurrence of variations in *CDKN2A* and *MC1R* genes. Clinical and phenotypic characteristics were also determined.

Materials and Methods

Subjects

32 unrelated patients (12 males, 20 females) of Greek-Cypriot origin with CM (31 sporadic and 1 familial) were referred from the Nicosia General Hospital in Cyprus. All patients were clinically diagnosed with CM according to the ABCD system followed by excisional biopsy and histological diagnosis. A total of 45 control subjects (19 males, 26 females) of Greek-Cypriot origin were also included in our study. Written consent was given prior to any genetic testing. Patient personal data were also collected by reviewing medical records and interviewing the patient using a standard questionnaire.

Amplification of CDKN2A and MC1R genes

Total genomic DNA samples were isolated from peripheral whole blood using GentraPuregene Blood Kit (Qiagen, Hilden, Germany). DNA sequencing was run from 100 ng genomic DNA, that were amplified using appropriately designed primers for the *CDKN2A* and the *MC1R* genes on Primer3 software version 0.4.0 (http://frodo.wi.mit.edu/). The *CDKN2A* gene primers covered all three exons and included two versions of exon 1 primers for variants p16INK4a (exon1alpha) and P14ARF (exon1beta)(see supplementary material). All PCR reactions with the above primers were run with 57°C annealing temperature for 30 cycles. The PCR products were analysed on an Applied Biosystems 3130xl Genetic Analyzer and the results were analyzed using Sequencing Analysis® 5.3 software (Applied Biosystems).

Statistical Analysis

Descriptive statistics were used to report the distribution of patients' demographic and clinical characteristics. Data are presented as mean ± SD (continuous variables) and percentages (categorical variables). Chi-square analysis was used to assess the association between patient characteristics and presence of *MC1R* mutations. Parametric (t-test) or nonparametric (Wilcoxon Rank Sum test) techniques were used as appropriate to assess differences in age between male and female melanoma patients. All analyses were performed using SAS, v.9.4 (SAS Institute Inc., Cary, NC, USA) software.

Results

Clinical data and patient characteristics

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Only 1 out of 32 Cypriot patients diagnosed with CM was familial. The others were all sporadic cases. Twenty of the patients were females (62.5%) and 12 cases were males (37.5%). Mean age at diagnosis was 58 (53.3±16.8 for female patients and 65.9±15.4. for males). According to the patients' medical history, only 4 cases (3 female and 1 male) were diagnosed with other types of cancer. More specifically, 2 patients had basal carcinoma cancer, 1 patient was diagnosed with prostate cancer and 1 patient had endometrial/ovarian cancer. Furthermore, Dysplastic Nevus Syndrome (DNS) was evaluated in 27 patients and according to our data, a total of 7 patients were diagnosed with DNS.
Further to clinical characteristics, phenotypic characteristics of patients were also assessed. More specifically, skin, hair and eye color was evaluated in 29 of the 32 patients. The vast majority of patients had fair skin (28 out of 29 cases) and only 1 patient had dark skin. Also, most patients had brown (16 cases) or black (9 cases)hair. Only 2 patients were blonde and 1 case had red hair. Eighteen patientshad brown eyes, 11 patients had blue/green and 3 caseshad black eyes. Freckling was detected in 18 patients.
To check for melanoma risk related environmental parameters that our patients may have been exposed to, they were asked to report the amount of sun exposure during childhood, amount of sun exposure during work, as well as their tanning ability. Our results (for 29 patients) showed that 16 patients had frequent sun exposure during childhood and 11 of these cases reported poor tanning ability with frequent sunburn. A total of 7 patients reported rare exposure during childhood and poor tanning ability. Overall, 20 of patients reported poor tanning ability. Finally, sun exposure during work was also assessed in patients. According to our data, the majority of the patients (13 cases) had an occupation of low sun exposure, whereas 6 cases had an occupation of high sun exposure.

Genetic profile of patients and controls

***CDKN2Ap.Ala148Thrvariant identified in Cypriot melanoma patients*:** As a first step we screened *CDKN2A*for variations. One common *CDKN2A*variation was identified in our patients (Table 1). The non-synonymous p.Ala148Thr found in *CDKN2A*exon 2, was detectedin 3out of 32 patients. This variant has been previously reported (Debniak *et al.* 2005). No other variations were detected in the remaining exons of *CDKN2A*in patients. The control group revealed no variations within the *CDKN2A* locus.

***Various MC1R variants detected in Cypriot melanoma patients*:** Following evaluation for *CDKN2A* variants, Cypriot melanoma patients were screened for *MC1R* geneticvariations. Altogether5different variants were detected in *MC1R* gene in different patients (Table 1).Four of these previously reportedknown non-synonymous variants were identified: p.Val60Leu, p.Val92Met, p.Arg142His, p.Arg160Trpin addition to thesynonymous: p.Thr314Thr. All *MC1R* variationswere observed in a total of 19 patients as follows:p.Val60Leu (11 cases), p.Arg142His (4 cases), p.Thr314Thr (3 cases), p.Val92Met (2 cases) and p.Arg160Trp (1 case). The combinations of p.Val92Met/p.Thr314Thr and p.Val92Met/p.Arg142His/p.Thr314Thr were detectedin 2 patients. Since no parental samples were available, the above variants could not be classified as chromosomally located *in cis* or *in trans*. It seems that variant p.Val60Leu, a common *MC1R* variant observed most frequently in many populations,isalsocommon among the Cypriot population, as it was detected with the highest frequency in both patients and controls. Overall, our results on*MC1R* screening indicate that several variations in this gene exist among Cypriot melanoma patients.

***Combination of CDKN2A p.Ala148Thr and MC1Rp.Arg142His variants found in 2 patients*:**Interestingly, after sequencing for both *CDKN2A*and *MC1R*, variants in both genes wereseen in 3 patients (Table 1). Two unrelated patients were found with the *CDKN2A* gene variant p.Ala148Thr in combination with *MC1R* p.Arg142His variant suggesting digenic inheritance.

Discussion

This work was the first attempt to investigate the genetic and phenotypic profile of CM patients of Cypriot origin. Due to the low incidence of melanoma within the Cypriot population, a small sample

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of only 32 patients diagnosed with CMwere included in this study. No significant associations between genetic variants and clinical or other risk factors could be observed(freckling (p=0.1426), skin color (p=0.4483), hair color (p=0.4157), eye color (p=0.0586), frequency of sunburn (p=0.0942). However, this outcome may be affected by the small size of our sample.

Following genetic screening, one common *CDKN2A*variantp.Ala148Thrwas identified in 3 patients. No*CDKN2A* variants were identified in the control group. Interestingly, compared to reported frequencies in other melanoma populations, Cypriots seem to have the highest frequency (Table 2). This variant has been identified with higher frequency in melanoma kindredsand less commonly in general populations (Hussussian*et al.* 1994; Harland *et al.* 1997). Also, several studies observed an association of p.Ala148Thr with high risk for developing melanoma (Debniak *et al.* 2005; Bakos *et al.* 2011). These results support its role to melanoma susceptibility. On the other hand, other studies concluded that p.Ala148Thr allele is not associated with the risk of CM (Spica *et al.* 2006). Furthermore, although initial functional studies suggested that this variant is a polymorphism, which seems to have no major influence on p16INK4a function (Ranade *et al.* 1995),other studies demonstratedits reduced effectiveness in inhibiting cell proliferation (Walker *et al.* 1999). Its role to melanoma susceptibility is also supported by results which demonstrate that p.Ala148Thr is in linkage disequilibrium with the 493A>T *CDKN2A* promoter variant, known to affect gene expression (Harland *et al*. 2000).Further assessment of p.Ala148Thr in other populations or in conjunction with other genetic variants,should further help elucidateits role in melanoma predisposition.

Further to investigating*CDKN2A,* we chose to screen *MC1R* gene for variations, mainly based on indications that *MC1R* variants significantly increase penetrance of *CDKN2A*mutations demonstrated by several studies (Demenais *et al.* 2010).A total of 5 previously reported*MC1R*variations were detected in our sample despite the small size of our sample, suggesting that *MC1R*has a high level of variability in Cypriot melanoma patients (Table 1).*MC1R*variationsidentified in our patients, were also identified in other populations as shown in Table 2. Compared to reported frequenciesofp.Arg142His in different populations, Cypriots seem to have the highest frequency (15.6%) (Table 2). However,our estimates may be affected by the small size of our sample. Only variant p.Val60Leu was detected in our controls (10 out of 45 cases) which, according to a review by Gerstenblith et al., is reported to be the most common *MC1R* variant across most populations (Gerstenblith *et al.* 2007). Reported findings on structural and functional characteristics on all variants and clinical significance are summarized in Table 3. Functional studies on several *MC1R* variations including p.Val60Leu, p.Arg160Trp, p.Val92Met have demonstrated slight or severe loss of MC1R function through dissimilar mechanisms (Garcia-Borron *et al.* 2005), thus providingsupporting evidence for a predisposing role of these variants in melanoma susceptibility. More specifically, functional studies suggested that missense variant p.Val60Leu may decrease activation of cAMP production (Schioth*et al.* 1999). Another study suggested that p.Val92Met may reduce binding affinity of MC1R for α-MSH (Ringholm*et al.* 2004). Also, p.Arg160Trp has been linked to a reduced functional coupling of MC1R and poor stimulation of cAMP production (Scott *et al.* 2002).Additionally, confirmed by meta-analysis, p.Arg142His and p.Arg160Trp have been associated with melanoma development (Raimondi *et al.* 2008).Interestingly, the *MC1R* combination p.Val92Met/p.Arg142His/p.Thr314Thr was detected in one patient who was also clinically diagnosed with basal cell carcinoma prior to developing melanoma, in contrast to the patient identified with the *MC1R* p.Val92Met/p.Thr314Thr combination who was diagnosed only with melanoma. This supports previous studies which concluded that two or more *MC1R* non-synonymous variants show an increased risk ofbasal cell carcinoma than those without non-synonymous variants (Ferrucci*et al.*2012). *MC1R* variant p.Thr314Thr is a synonymous variant and according to RNAsnp webserver (http://rth.dk/resources/rnasnp/), no SNP effects have been identified on the RNA secondary structure of this variant(p-value = 0.2264).Finally, in our study, 2 of the patients were carriers of both *CDKN2A* p.Ala148Thr and *MC1R* p.Arg142His, suggesting digenic inheritancethat may give evidence of different gene variants acting synergistically to contribute to melanoma development.

In conclusion, previously described variations in *CDKN2A* and *MC1R* are found to exist in the mutation spectrum of Cypriot patients with CM. This study contributes to the investigation of genetic

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variants suspected to have a role in susceptibility to melanoma, across different populations. Whether these genetic variants may play a role in CM is still unclear. Further assessment of these variants should help elucidate their role in melanoma predisposition among Cypriots.

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Tables

Table 1.Variations detected in Cypriot melanoma patients and control group using genomic sequencing.

Gene	Variations	No. Patients (%) (n=32)	No.Controls (%) (n=45)
CDKN2A	p.Ala148Thr/c.442G>A	3 (9.4)	0
MC1R	p.Val60Leu/c.178G>T	11 (34.4)	10 (22.2)
	p.Arg142His/c.425G>A	4 (12.5)	0
	p.Arg160Trp/c.478C>T	1 (3.1)	0

	p.Thr314Thr/c.942A>G	1 (3.1)	0
	p.Val92Met/c.274 G>A, p.Thr314Thr	1 (3.1)	0
	p.Val92Met, p.Arg142His, p.Thr314Thr	1 (3.1)	0
CDKN2A & MC1R	p.Ala148Thr(*CDKN2A*)/p.Arg142His(*MC1R*)*	2 (6.2)	0
	p.Ala148Thr(*CDKN2A*)/p.Val60Leu(*MC1R*)	1 (3.1)	0

[1]

Table 2.Reported frequencies of *CDKN2A* p.Ala148Thrand four non-synonymous *MC1R*genetic variants in melanoma populations of various geographical areas.

Country	**Frequency (%) *CDKN2A* genetic variants p.Ala148Thr**				**Reported study**
Cyprus	**9.4**				Current study
Brazil	2.6				(Bastos *et al.* 2011)
Iceland	2.5				(Goldstein *et al.* 2008)
Latvia	6.0				(Pjanova *et al.* 2007)
Italy	6.7				(Spica *et al.* 2006)
France	3.4				(Spica *et al.* 2006)
Poland	7.0				(Debniak *et al.* 2005)
	Frequency (%) *MC1R*genetic variants				
	p.V60L	**p.R142H**	**p.R160W**	**p.V92M**	**Reported study**
Cyprus	34.4	**15.6**	3.1	6.3	Current study
Spanish	29.9	3.3	6.2	**11.7**	(Puig-Butille *et al.* 2013)
Australia	12.3	1.2	10.1	9.6	(Cust *et al.* 2012)
US	17	1.4	1.8	7	(Council *et al.* 2009)
Greece	**36.6**	5.7	4.1	8.1	(Stratigos *et al.* 2006)
Italy	30.3	3.6	5.5	7.3	(Landi *et al.* 2005)
France	16.7	1.4	7.9	5.6	(Matichard *et al.* 2004)
Netherlands	10.2	1.2	**14.2**	10.2	(Kennedy *et al.* 2001)

[2]

Table 3.Detected variant properties.

Variant	**Variation Type, General Population Allele Frequency (ExAC*)** **Clinical Significance** **Structural and Functional Characteristics**
CDKN2Ap.Ala148Thr	Missense (nonpolar to polar), 2% Benign variant (ClinVar)** Demonstrated reduced effectiveness in inhibiting cell proliferation (Walker *et al*. 1999)

[1] * p.Ala148Thr(*CDKN2A*)/p.Arg142His(*MC1R*), a combination seen in 2 patients, suggests digenic inheritance.

[2]Only variants detected among Cypriot patients are presented. Additional*CDKN2A*or *MC1R* variants identified in other populations are not shown. Highest reported frequency for each variant, is shown in bold.

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	Associated with melanoma development (Debniak *et al.* 2005; Bakos *et al.* 2011)
MC1R p.Val60Leu	Missense (nonpolar to nonpolar), 8.3% Probably damaging variation (predicted using PolyPhen-2***) Close to cAMP recognition site Associated to fair/blonde and light brown hair colours (Box *et al.* 1997) Demonstrated reduced activation of cAMP production (Schioth *et al.* 1999)
MC1Rp.Val92Met	Missense (nonpolar to nonpolar), 7.6% Benign variation (predicted using PolyPhen-2) Alters alpha-helix structure of the second transmembrane domain (Valverde*et al.* 1995) Seems to reduce MC1R binding affinity for a-MSH (Ringholm *et al.* 2004)
MC1Rp.Arg142His	Missense (positive to positive), 0.5% Probably damaging variation (predicted using PolyPhen-2***) Located in the second intracellular loop (coupling region) Seems to reduce activation of cAMP production (Schioth *et al.* 1999) Associated with melanoma development (Raimondi *et al.* 2008)
MC1Rp.Arg160Trp	Missense (positive to nonpolar), 5.0% Possibly damaging variation (predicted using PolyPhen-2) Demonstrated reduced functional coupling of MC1R/poor stimulation of cAMP production (Scott *et al.* 2002); Associated with melanoma development (Raimondi *et al.* 2008)
MC1Rp.Thr314Thr	Synonymous, 15% Located in C-terminus Appears with highest frequency in African, Asian-Indian, and Papua New Guinean populations (Gerstenblith*et al.* 2007)

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[3] * General population allele frequencies were obtained using ExAC Browser (http://exac.broadinstitute.org/gene/ENSG00000165731)
** Clinical significance of variation was based on ClinVar reports (http://www.ncbi.nlm.nih.gov/clinvar/)
*** Prediction of possible impact of the amino acid substitution on the protein structure and function was based on PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/)