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Benefits of oral *Polypodium Leucotomos* extract in MM high risk patients

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INTRODUCTION

Melanoma (MM) is the human malignancy that has undergone the greatest increase in incidence during the last few decades. To improve prognosis, it is critical to identify patients bearing high risk of suffering MM, and as well as genetic factors of predisposition and progression. Ninety percent of melanomas are considered sporadic, and the main risk factors implicated are ultraviolet radiation (UVR) and the presence of melanocytic nevi [1–5]. Exposure of human skin to sunlight containing both A and B UVR leads to deleterious cutaneous effects, the most remarkable being skin cancer [6]. Currently the most widely used method of protection against UV-induced damage is the use of topical sunscreens, which act through physical particles enriched with chemical molecules that reflect and/or absorb UVR. A systemic photoprotective agent would have several advantages over topical protection, as this would provide more uniform, prolonged, and total body surface protection, independently of the specific properties of the creams, the amount applied, and other individual factors such as sweating or water bathing.

Polypodium Leucotomos (PL) is a tropical fern that has long been used for the treatment of inflammatory disorders by Native Americans [7]. Extracts of PL, topically applied or orally taken, have been shown to have a variety of potentially beneficial properties. Administration

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of PL to mice decreases the degree of histological parameters of photoaging damage resulting from UVB radiation exposure and lowers the incidence of UVB radiation-induced non-MM skin cancers [8]. Furthermore, PL extract activates tumor suppressor p53, inhibits UV-induced Cox-2 expression, reduces inflammation, enhances the removal of UV-induced photoproducts, such as cyclobutane pyrimide dimers, as well as reduces oxidative DNA damage and decreased UV-induced mutagenesis [9]. Finally, oral administration of PL extracts to humans decreases ultraviolet radiation-induced skin damage [10, 11] as well as psoralen- UVA radiation-induced phototoxicity and pigmentation [10, 12] and, recently, also has shown to prevent UVA-induced common deletions and mitochondrial DNA damage (13).

Heritable alleles for MM susceptibility range from high-risk genes, high penetrance alleles that are rare, to low-risk genes, low-penetrance alleles that are rather ubiquitous. This has been captured in the adage “common variants (i.e., polymorphisms) cause common disease (ie, sporadic MM), whereas rare variants (i.e., mutations) cause rare disease (ie hereditary MM).” The high penetrance alleles can be responsible for rare familial clusters of MM, but, fortunately, they do not participate in common sporadic cases.

Low penetrance alleles are quite prevalent in the general population, but are not as closely associated with the ultimate development of MM. Thus, whereas more patients with MM have a combination of these low-risk allelic mutations, most patients with these variants will not ever develop MM.

The cyclin-dependent kinase (CDK) inhibitor 2A gene (*CDKN2A*) is the best-established high-risk locus for MM. It has been determined that, in aggregate, 25% to 50% of familial MM kindred are affected by a *CDKN2A* mutation. This prevalence increases as the number of affected cases increase in the index family. In smaller studies, up to 10% of patients with multiple primary MM (MPMs) have been identified to display a *CDKN2A* mutation. In a large population-based study, however, reported *CDKN2A* mutation rates are about 1% for the unselected MM patient and about 3% for individuals with MPM [14, 15]. Although most familial cases of MM are caused by mutations in *CDKN2A*, some families are affected by genetic mutations downstream of *CDKN2A*. Mutations in cyclin-dependent protein kinase 4 (*CDK4*) have been identified in some MM kindreds [16].

Beyond genes known to confer a high degree of susceptibility to cutaneous MM, other genes have been proposed to confer moderate risk. Fair skin and red hair colour has been associated with increased MM risk. Specific variants in the Melanocortin 1 receptor (*MC1R*) gene produce variable quantity of the red/yellow pheomelanin pigment which induces oxidative cell-damage, instead of the brown to black eumelanin which is photoprotective. Depending on these *MC1R* variants, and other pigment track genes (*OCA*, *TYR*, …) the most common phenotype is that of individuals of blond or red hair, Fitzpatrick’s skin phototype I, tendency to sun-induced freckling, and reduced tanning response [17, 18]. Variants in *MC1R* are relatively common in the white population and have been proposed to confer low to moderate MM susceptibility risk [19, 20]. One large study noted a 2.2-fold increased in the relative odds of developing cutaneous MM among individuals with one “red hair” variant (RHV), and a 4.1-fold increased relative odds in those with two variants. Increased risk

remained unchanged for carriers who had non-RHV and darker skin. RHV of *MC1R* also have been demonstrated to increase the rate of MM in individuals who have *CDKN2A* mutations [21, 22]. More than 30 allelic variants of the human *MC1R* have been identified, mainly in Northern European populations and in Australia. The consequences of these variants on physiological function of the product of the *MC1R* gene have just begun to be elucidated. For example, it is known that R160W homozygote and R151C/D294H, R160W/D294H compound heterozygote fail to couple to cAMP activation, show impaired tyrosinase activation in response to αMSH stimulation and display a pronounced sensitivity to UVR [23].

The aim of this study was to test the possible role of an oral PL extract to improve systemic photoprotection in patients at risk of skin cancer. The first goal was to further analyze the ability to decrease UV-induced erythema. A second aim was the study of the interaction among *MC1R* polymorphisms and *CDKN2A* status with the minimal erythematous dose (MED) and their influence in the response after oral PL.

PATIENTS AND METHODS

Participant selection

We included **61 patients** belonging to the following groups: **25** patients with familial and/or multiple MM (2 or more first-degree relatives with MM and/or two or more MMs in the same patient), **20** patients with sporadic MM and **16** patients with atypical mole syndrome without history of MM.

Patients were included after they read and signed a written informed consent form approved by the ethical committee of Hospital Clinic de Barcelona, Barcelona, Spain. Exclusion criteria were history of abnormal photosensitivity (defined by any photo-induced dermatosis or demonstration of a decreased minimal erythemathous dose to UVB; lower than 100mJ/cm2), or photosensitivity induced drugs intake, UVR (natural or artificial) exposure 6 weeks before the study.

Descriptive study

Patient phenotyping—Skin type of each individual was determined in accordance to Fitzpatrick classification (I to IV), eye colour was categorized as “brown”, “black”, “green” or “blue” and hair colour was recorded as “brown”, “black”, “blonde” or “red”. Number and clinical and dermoscopical description of nevi was studied.

Patient genotyping—Blood samples were taken from all patients. The PUREGENE DNA Isolation Kit (Gentra Systems, Minneapolis, MN, USA) was used to isolate genomic DNA from lymphocites according to the manufacturer's instructions.

Promoter (−34G>T variant), intronic (IVS2–105) and coding regions of the *CDKN2A* gene (exons 1α, 2 and 3 of the p16INK4A protein and exon 1β corresponding to p14ARF protein) were amplified by PCR using primers and conditions previously described [24]. *MC1R* was amplified using primers described by Chaudru et al.[25]. SSCP analysis was carried out. Samples with abnormal migration products were sequenced as follows: PCR products were

purified using the GFX™ PCR DNA and Gel Band purification kit (Amersham Biosciences) and automatically sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA) and an ABI3100 automatic sequencer (Applied Biosystems).

UVR sensitivity assessment—UVB minimal erythematous dose (MED) was assessed in each patient by means of a Waldmann UV800 lamp as performed in The Fotobiology Unit of the Hospital Clinic, Barcelona. Normal skin on the central back of patients was irradiated in 6 consecutive increasing dose-exposure windows (doses of 0.02, 0.05, 0.07, 0.10, 0.15 and 0.20 J/cm^2) on a 2-cm^2 area at 20 cm distance from the lamp (Fig 1). The UVB-MED was defined as the minimal dose of radiation per cm^2 which induces confluent erythema at 24 hours with 4 sharp borders of the exposed skin site.

Intervention assay design—After basal MED assessment, all participants received the same oral dose of a commercial form of PL (120mg each capsule; Difur®, Industrial Farmaceutica Cantabria, SA, Madrid, Spain); **720 mg** of oral PL in three doses, (240mg every 8hours) and **360 mg** in a single dose, were administered one day and 3 hours respectively, before a second MED assessment (total dose of **1080mg**).

Clinical evaluation of both basal and post-treatment MED were performed by 2 experienced dermatologists.

Statistical analysis

Descriptive analysis of the sample was performed, including percentages for categorical variables, and mean, minimum, maximum and standard deviation values for continuous variables. Comparisons of continuous variable means were performed using Student's exact t test when variables followed a normal distribution. Comparisons of discrete variable means were performed using the Mann–Whitney non parametric test. Comparisons between categorical variables were performed with χ^2 tests and Fisher corrections were performed when required. Categorical multivariable analysis was performed to evaluate independent risk factors of reduced MED after oral treatment with PL extract using a stepwise forward approach.

RESULTS

Descriptive results

Phenotyping: A total of **61** patients were included in the study belonging to the following groups: 20 (33.3%) sporadic MM, 25 (41.7%) familial MM and 16 (25%) dysplastic nevus syndrome. The mean age was 47.79±14.75 years with a minimum of 15yr and a maximum of 76yr. Gender distribution was 29 male (48.3%) and 31 female (51.7%). Individuals from our study population mainly exhibit a dark eyes and hair phenotype: 75.9% dark hair and 63.8% brown eyes. However, the most frequent skin type was II (47.4%) followed by skin type III (36.8%). Nevertheless, a non-depreciating percentage of our patients showed at least one phenotypic risk factor: 56.1% of individuals showed fair skin pigmentation, 36.2%

green or blue eyes, 24.1% red or blonde hair (10.3% red hair) and 53.5% accumulated photodamage such as actinic lentigos (Table 1).

Study of mutations on the *CDKN2A* gene were performed in 37 individuals. Of these, 9 (24.3%) were mutation carriers, all of them belonging to the familial melanoma group. From the familial MM patients, 9 (33%) were mutation carriers. Fifty five patients were studied for polymorphism in the *MC1R* gene of whom 17 (30.9%) were wild type (WT). Twenty seven patients were carriers of one functional variant, 9 patients were carriers of two functional variants and 2 patients were carriers of three functional variants of the *MC1R* gene. Three patients were carriers of two red hair variants in the *MC1R*. The distribution of variants on our sample is shown in Table 2.

Basal photosensitivity—The **mean basal MED** in overall population was 0.124 ± 0.04 J/cm^2, with a minimum of 0.07 and a maximum of 0.20 J/cm^2. **Interestingly, men and women presented differences in basal MED values**. Women had a higher basal MED than men (0.14 vs 0.11 J/cm^2), U=232.5, p<0.05. As well, a high percentage of patients with familiar MM had a higher basal MED value (≥0.15 J/cm^2) than the rest of the patients (sporadic melanoma and dysplastic nevus syndrome), but this was not statistically significant. Nor differences in basal MED were found between patients with past history of MM (N=45); also, differences were not statistically significant when we compared with patients with dysplastic nevus syndrome without MM history (N=16),(0.127 J/cm^2 vs 0.117 J/cm^2, U= 293; p=0.23). None of these depended of presence of actinic lentigos, hair colour, eye colour, or *MC1R* polymorphisms (Table 3).

Despite the lack of statistically significant associations between a lower basal MED value and having red hair variants (RHV), non RHV or wild type (WT) *MC1R*, we observed that patients with at least one RHV had a tendency to lower basal MED values.

Photosensitivity after treatment—The mean of MED after PL treatment was 0.161± 0.047 J/cm^2, with a minimum of 0.07 and a maximum of 0.3 J/cm^2. Again, we found significant different MED values after PL depending on gender, with a higher post-PL MED in women than men (0.18 vs 0.14 J/cm^2), U=286, p<0.05.

Importantly, oral **PL treatment significantly increased the MED mean in all group patients (0.123 to 0.161 J/cm^2, p<0.05)**.

We noted that patients without history of MM tended towards higher MED post PL value (≥0.15 J/cm^2) than patients with MM (93.3% vs 77.8%), but this was not statistically significant. Also, we found no significant differences in MED values after PL treatment between patients with and without history of MM (0.167 vs 0.157 J/cm^2, U=326.5, p=0.83). We did not observe differences depending on the presence of actinic lentigos, the hair colour, the eye colour, or *MC1R* variants.

The effect of PL in increasing the mean MED was similar in all patients, independently of the group (with vs without MM, patients with familial MM vs without familial MM). The treatment was effective in terms of increasing mean MED in all groups. Although not

significant, we noticed a stronger effect of PL on the MED of patients with familial MM compared to those with MM (U=273, p=0.06). Among the patients with familial MM, those exhibiting a mutated *CDKN2A* and/or polymorphisms in *MC1R* displayed larger differences in response to treatment with PL.

Of the 61 patients studied, 21 (35%) did not show any effect in MED value after orally administered PL, whereas an increase in the MED values was demonstrated in the other 40 (65%) patients. The increased in the MED after PL was associated with **dark eyes** (χ^2=4.67, p<0.05) (OR 4.47, CI 95% 1.22–16.34) and a **lower basal MED** value (χ^2=6.90, p<0.05) (OR 4.59, CI 95% 1.23–7.47). Phenotypic characteristics of responders and non responders are shown in Table 4. Multivariable analysis adjusted for age and sex revealed dark eyes and a lower basal MED to be independent risk factors for increasing MED after treatment. In this way, having dark eyes and a higher sensibility to UVR may be predictors of a good response to PL.

DISCUSSION

This study demonstrates that oral administration of PL leads to a significant reduction of sensitivity to UVR in terms of increased UVB - MED values (p<0.05). This improvement in sensitivity to UVR could be observed in all the groups included in this study, that is patients with dysplastic nevus syndrome, patients with sporadic MM and patients with familial MM.

MM is the most devastating form of skin cancer. The steady increase in the incidence of MM and its resistance to chemotherapy, together with its high potential to metastasize, have emphasized the importance of its prevention. It is becoming clear that solar UVR is a main risk factor in the aetiology of MM [26] and its protection is the only primary prevention against MM development. Besides avoiding sun exposure, using a sunscreen is the most accepted photoprotection method in developed countries, and the preventive effect of using a sunscreen in non-MM skin cancer has often been suggested. However, there is still debate whether it provides adequate protection against MM and nevus induction. Inadequate sunscreen application has been found to be a common failing leading to deficient photoprotection [27, 28]. There is increasing evidence that a number of substances when taken orally exert a preventive effect against UV-induced skin damage without adverse effects. The mechanisms of action are highly varied, affecting diverse signaling pathways and resulting in an antioxidant, anti-inflammatory, and immunomodulatory activity [29]. A systemic photoprotective agent would have an advantage over topical protection, as this would provide uniform, total body surface protection. Besides, patients with familial melanoma have other risk factors, which represent the main burden of risk, such as mutations in *CDKN2A* gene. Certain allelic variants in *MC1R* gene, namely R151C, R160W and D294H, are strongly associated with red hair phenotype and increased melanoma susceptibility. Natural expression of two of these variants sensitizes melanocytes to the citotoxic effect of UV, and increases the burden of DNA damage and oxidative stress [30]. In this study, patients with red hair variants of *MC1R* had no statistically significant lower basal MED values than patients without red hair variants, but we found a trend to lower basal MED values in patients with at least one red hair polymorphism. It could be possible that the number of patients included in the study is insufficient to achieve statistical

significance. Moreover, patients with *wild type* MC1R had not higher basal MED values than patients with not red hair *MC1R* polymorphisms. Interestingly, a high percentage (56%) of our patients with familial melanoma, have a high basal MED value (MED≥0.15 J/cm^2), and they did not bear *MC1R* polymorphisms or red hair polymorphisms. In these familial melanoma cases the risk is probably most associated to high penetrance genes like *CDKN2A* and less to low medium penetrance genes like *MC1R* or other genes related with fair skin. Despite of these findings, the exposition to RUVB is a modifying environmental factor that increases the risk of melanoma also in this context [31]. The results of this study are very promising and suggest that patients with familial melanoma can benefit their selves from orally PL as far as it reduces significantly sensitivity to RUVB in terms of increased MED UVB ($p<0.005$). Due to the intrinsic high predisposition of these patients, any positive intervention to reduce their risk is very positive. Also very interesting and promising is the fact that orally PL reduces significantly RUVB effect in patients with sporadic MM and patients with dysplastic nevus syndrome in which the effects of UVR are the main responsible in the melanoma genesis.

Analysis of the basal MED values by gender reveals that women had a higher basal MED value than men, which means a lower sensitivity to UVR. No other differences between groups were observed although patients with red hair polymorphisms in *MC1R* had lower basal MED values than patients without, but this was not statistically significant (Table 3). The same results were found when analysing MED values post treatment, women had statistically significant higher MED values post treatment than men.

We found that patients exhibiting a positive effect of PL had more frequently dark eyes and a lower basal MED. The multivariable analysis adjusting by age and sex showed that dark eyes and lower basal MED are independent factors to predict a better response to PL.

Therefore, we can conclude that dark-eye patients and patients with higher UVR sensibility (lower basal MED) would be the most benefited from oral PL treatment. Previous studies have demonstrated that a single dose of orally administered PL decreases UVR-induced damage of human skin [10, 11]. However, the present study pioneers the effect of orally administered PL in patients with high risk to develop MM. Our results are very promising and suggest the need to perform long-term follow-up and long term administration of PL studies in patients bearing high risk of developing MM.

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ABBREVIATION AND ACRONYM LIST

MM	malignant melanoma
UVR	ultraviolet radiation
PL	*Polypodium leucotomos*
MED	minimal erythematous dose
CDKN2A	cyclin-dependent kinase (CDK) inhibitor 2A gene
MPMs	multiple primary MM
MC1R	Melanocortin 1 receptor

Table 1

Distribution of studied variables in men and women.

	Men	Women	Whole sample	
	N= 29 (48.3%)	N= 32 (51.7%)	N=61 (100%)	
MM	N= 8 (40%)	N= 12 (60%)	N=20 (33.3%)	
Familial MM	N= 13 (52%)	N= 12 (48%)	N=25 (41.7%)	
Dysplastic nevus syndrome	N= 8 (50%)	N= 8 (50%)	N=16 (25%)	
Mean age	50.03y	45.75y	47.79y	0.261
Skin type I–II	N= 15 (53.6%)	N= 17 (58.6%)	N=32 (56.1%)	0.701
Skin type III–IV	N= 13 (46.4%)	N= 12 (41.4%)	N=25 (43.9%)	0.701
Dark hair	N= 22 (78.6%)	N= 22 (73.3%)	N=44(75.9%)	0.641
Blond/Red hair	N= 6 (21.4%)	N= 8 (26.7%)	N=14 (24.1%)	0.641
Dark eyes	N= 20 (71.4%)	N= 17 (56.7%)	N= 37 (63.8%)	0.242
Clear eyes	N= 8 (28.6%)	N= 13 (43.3%)	N=21 (36.2%)	0.242
Actinic lentigos (moderate-severe)	N= 12 (42.9%)	N= 18 (62.1%)	N= 30 (52.6%)	0.146

Table 2

Distribution of the *MC1R* variants.

Variant	Frequency	%
D294H	6	12.5%
R151C	12	25%
R160W	3	6.3%
R163Q	2	4.2%
F45L	1	2.1%
Q233Q	1	2.1%
V60L	12	25%
V92M	5	10.4%
Y152X	1	2.1%

Table 3

Basal MED means comparison between groups

Group	Basal MED mean	*U*	p
Lentigos (N=30) vs no lentigos (N=26)	0’123 vs 0’124 J/cm^2	401	0’945
Dark eye color (N=37) vs light (N=21)	0’120 vs 0’128 J/cm^2	348’5	0’486
Dark hair color (N=44) vs fair (N=14)	0’122 vs 0’123 J/cm^2	308	1
MC1R polymorphisms yes (N=38) vs no (N=17)	0’120 vs 0’123 J/cm^2	309	0’784
RH polymorphisms yes (N=19) vs no (N=36)	0’114 vs 0’125 J/cm^2	297	0’398
Men (N=29) vs Women (N=32)	**0’107 vs 0’140 J/cm^2**	**232’5**	**<0’05**
MM (N=45) vs no MM (N=16)	0’127 vs 0’116 J/cm^2	293	0’238
CDKN2A (N=9) vs WT (N=28)	0’122 vs 0’118 J/cm^2	302	0’795

Table 4

Phenotypic characteristics of responders and non-responders.

	SKIN TYPE	HAIR COLOUR	EYE COLOUR
RESPONDERS	I N=3 (7.9%) II N=17 (44.7%) III N=15 (38.5%) IV N=3 (7.9%)	Dark N=28 (73.7%) Blond-red N=10 (26.3%)	Dark N=28 (73.7%) Blue-green N=10 (26.3%)
NON RESPONDERS	I N=2 (10.5%) II N=10 (52.6%) III N=6 (31.6%) IV N=1 (5.3%)	Dark N=16 (80%) Blond-red N=4 (20%)	Dark N=9 (45%) Blue-green N=11 (55%)