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Familial Melanoma in Italy: A Review

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1. Introduction

Looking back to history, the first accredited description of melanoma appeared in the writings of Hippocrates (5th century, BC) as a "fatal black disease"; over the centuries, several other physicians have described pigmented malignant skin lesions, but only during the 1800s significant gains were obtained in the comprehension and treatment of melanoma. In 1820 William Norris suggested for the first time a genetic basis for the disease, reporting the development of a skin neoplasm in a father and his son, and in 1838 Carswell used the medical term "melanoma" to describe pigmented lesions.
Cutaneous melanoma represents a malignant skin cancer, which arises from the neoplastic transformation of specialized pigment-producing cells, the melanocytes. Its aggressive features, in terms of tendency to develop metastasis and strong resistance to therapy, make melanoma one of the deadliest forms of cancer.

1.1 Origin and pathogenesis

During embryogenesis, cellular precursors emerge from the neural crest and migrate to various sites, as uveal tracts, meninges, ectodermal mucosa and skin, where they finally establish to the epidermal-dermal junction, where they differentiate into melanocytes (Bennett, 1993), making dendridic contacts with the basal keratinocytes and thus forming the so called "epidermal melanin unit". The melanin produced by the melanocytes is transferred to keratinocytes for the adsorption and scattering of light radiation. The quantity of pigment dictates skin pigmentation and protection degree from UV-induced damage.

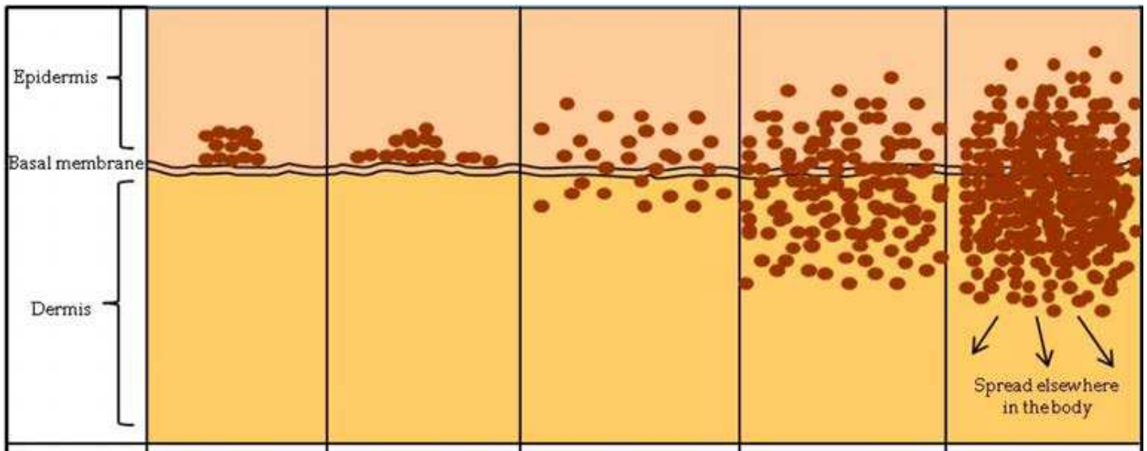
1.1.1 Melanoma stages

As reported in Figure 1, the pathogenesis of melanoma is characterized by a series of histological and molecular alterations; in particular, five distinct stages have been proposed for melanoma development (Chin, 2003): (I) acquired and/or congenital nevi represent benign forms of melanocytic proliferation, different from (II) dysplastic nevi with some degree of structural atypia and a deeper disorder in the melanin unit organization; both benign and dysplastic nevi may evolve to (III) radial growth phase melanoma, characterized

by a lateral growth, that remains mostly limited to the epidermis, due to the dependence of melanocyte proliferation from growth factors released by keratinocytes (*in situ* melanoma). Subsequently, melanoma can shift to (IV) vertical, mitogens- and anchorage-independent, growth phase, with dermis and subcutaneous tissue invasion, that, further on, may give rise to a (V) fully metastatic melanoma. These stages, describing melanoma natural history, have corresponding molecular counterparts, providing a biochemical basis for the main cellular events responsible for melanomagenesis. Accordingly, mutations in genes, normally regulating proliferation, differentiation and cell death, are involved in all of the main steps of melanoma progression, as briefly summarized in Figure 1.

1.1.2 Clinical forms

The vast majority of melanomas arise from cutaneous sites and different clinical forms have been described so far. Superficial spreading melanoma, most common in 30-50 year-old patients, spreads horizontally with an early flat appearance, and irregular pigmentation and margins; lentigo maligna melanoma is typically located on sun-exposed sites (head, neck and arms) and presents large (3-4 cm in diameter) and irregular lesions with a very slow growth over 5-20 years; nodular malignant melanoma develops from dark and uniformly coloured lesions, which may ulcerate and bleed, it lacks the radial growth phase and can result in a rapid invasion of the dermis. These three types account together for approximately 85% of all melanoma cases. Other less frequent forms include desmoplastic melanoma, with a highly variable clinical appearance, mucosal lentiginous melanoma and acral lentiginous melanoma, the most common type in dark-skinned populations (Africans and Hispanics).



Histological stage	Congenital and/or acquired nevus (I)	Atypical/dysplastic nevus (II)	Radial growth phase (III)	Vertical growth phase (IV)	Metastatic melanoma (V)
Molecular alterations	Mutations in: BRAF NRAS MC1R alleles	Loss of CDKN2A PTEN CDK4 mutations	Decreased: SILV MLANA TRPM1 E-caderin expression	Expression of: N-caderin αVβ3 integrin MMP2 E-caderin loss	Additional events

Fig. 1. Melanoma natural history.

Five proposed stages for melanoma development and corresponding molecular events involved in malignant melanoma pathogenesis. (MC1R: *M*elano*C*ortin 1 *R*eceptor; CDKN2A: *C*yclin-*D*ependent *K*inase i*N*hibitor 2*A*; PTEN: *P*hosphate and *TEN*sin homolog; CDK4: *C*yclin *D*ependent *K*inase 4; MLANA: *MeLAN*in-*A*; TRPM1: *T*ransient *R*eceptor *P*otential cation channel sub-family *M* member 1; MMP2: *M*atrix *M*etallo*P*roteinase 2).

1.2 Melanoma epidemiology and etiology

It was reported that melanoma incidence rose during the last century worldwide. In recent decades, the annual increase in incidence rate was reported to be in the order of 3-7% for fair skinned Caucasians (Armstrong & Kricker, 1994) and to vary among populations with age, gender (Jemal et al., 2001) and geographical area. Melanoma is the third more frequent cancer in 30-45 years age group, with a higher occurrence in female and in fair-skinned populations living in sunny areas. Some dermatologists questioned whether this rising in melanoma incidence could reflect a real increase of disease occurrence or if it could be ascribed to a more intensive surveillance. Undoubtedly, implementation of screening programs has allowed the early detection of a larger number of pigmented and potentially suspect skin lesions (Dennis, 1999), with a corresponding larger number of new cases per year, that could, at least in part, justify the overall incidence increase. Moreover, some initially unsuspected lesions can be correctly diagnosed as true malignant melanomas (the so called "incidental melanomas") later on by histo-pathological analysis, because of persisting diagnostic limitations for very early lesions (Swerlick & Chen, 1997). Importantly, despite a higher incidence, melanoma mortality remains stable over the years.

Italy is considered a low melanoma incidence country, with geographical variations according to a decreasing North-South gradient, as reported by the Italian Cancer Registries. Melanoma incidence rates in Italy were reported to be of 8.5 cases per 100,000 in male and 10.2 case per 100,000 in female (Boi et al., 2003).

1.2.1 Risk factors

Melanoma epidemiology involves not only incidence and mortality rates, but also possible causal factors. Melanoma is characterized by a complex etiology, concerning both constitutional and environmental factors, that mostly in combination determine the likelihood of developing the disease. There is no question that sun exposure is the major environmental risk factor (Oliveria et al., 2006). Several data from epidemiological analysis on migration and geographical residence showed an increased risk in sunny areas, especially for individuals exposed during childhood and adolescence, both crucial ages for subsequent melanoma development (Autier et al., 1997; Robsahm & Tretli, 2001). Conflicting evidence, however, were reported to this end, since several studies did not confirm the role of childhood/adolescence as "critical time periods" (Kaskel et al., 2001; Pfahlberg et al., 2001). Intermittent sun exposure and sunburns, in particular, appear to be relevant factors as well (Gandini et al., 2005a).

To date, limited data are available on melanoma risk factors in Mediterranean populations, one of the largest Italian collaborative case-control study investigated the role of different potential risk factors, comprising modalities of sun reaction and history of sunburns (Naldi et al., 2000). Moreover, an Italian research group recently reported a protective effect of the so-called Mediterranean diet on cutaneous melanoma onset (Fortes et al., 2008).

On the other hand, several constitutional risk factors were shown to be involved in melanomagenesis. Phenotypic traits, including skin phototype and pigmentation (fair

complexion and sensitivity to sunburns), presence of freckles and light-coloured eyes are additional examples of host-related risk factors (Gandini et al., 2005b). Several evidence suggested that while the number of melanocytic nevi can represent a proper melanoma predictor, the presence of atypical nevi may assume an independent role (Bataille et al., 1998; Tucker et al., 1997). Thus, melanoma risk raises with nevus number and clinical atypia degree, with the highest risk for individuals presenting multiple atypical nevi (MacKie et al., 1993).

1.2.2 Familial melanoma

Probably, the most important and well documented host-related risk factor is familial history of melanoma, defined by the presence of two or more relatives affected by melanoma within a family branch. Familial melanoma accounts for approximately 5-10% of all melanoma cases and segregation analysis revealed an autosomal dominant mode of transmission. Moreover, likewise other inheritable cancer syndromes, familial melanoma is characterized by peculiar features (Kopf et al., 1986) including early age of onset, multiple primary melanomas and association with other cancers, namely pancreatic carcinoma in the so-called "Familial Melanoma/Pancreatic Cancer Syndrome" (Lynch et al., 2002). On the other hand, as for histological and clinical aspects, familial melanoma is indistinguishable from sporadic cases. Even though exposure to common environmental risk factors cannot be excluded, familial aggregation can be prevalently attributed to shared genetic factors and several melanoma-predisposing genes were identified so far. A crucial aspect of familial melanoma, as well as any cancer syndromes, is that what is inherited is a genetic predisposition, i.e. a greater risk, rather than the disease itself. The reason is that a mutated allele of a specific cancer-predisposing gene is constitutively present in every cell of the body (Knudson's "first-hit"). Thus, individuals belonging to melanoma-prone families, once carriers of genetic predisposition, have a markedly higher risk to develop melanoma during their lifetime, compared to the general population.

Aim of the present review is the description of the genetic basis of familial melanoma, the role of genetic counseling in its early diagnosis, the laboratory assessment of pathogenic gene mutations and an overview of the main studies and research investigations performed on familial melanoma in Italy.

2. Genetic basis of familial melanoma

During oncogenesis, both inherited and/or acquired genetic alterations take part in a multistep process that finally results in an invasive and metastatic neoplastic growth. Hereditary cancer syndromes, in particular, represent a model for the analysis of how germline mutations of specific genes can modulate cancer risk and hence influence the development of disease.

Several genes involved in familial melanoma susceptibility were reported and recent advances in molecular genetics, including genome-wide association studies (GWAS), may identify additional melanoma-predisposing alleles in the future.

2.1 High risk genes

The identified melanoma susceptibility genes include rare high risk and more common, moderate/low risk genes. It is important to point out that there is not an absolute distinction, in terms of conferred risk, between these two categories, but most likely a continuous gradient of gene effects, leading to a variable (from strong to weak) melanoma risk.

2.1.1 CDKN2A

The first real clues about the existence of a major melanoma susceptibility gene came from molecular cytogenetic evidence. Melanoma cell lines were found to have frequent deletions of the 9p21-p22 chromosome region and linkage with markers at 9p13-p22 was subsequently reported in several melanoma families. Some years later, a combination of tumour deletion and recombination mapping studies in melanoma families was used to further limit the position of a candidate gene that was then cloned, sequenced and recognized to be identical to a previously characterized cell cycle regulatory gene. This gene was variably called INK4A, CDK4I or MTS1, but designed by now as CDKN2A (for *C*yclin-*D*ependent *K*inase i*N*hibitor *2A*) by the Human Genome Organization Nomenclature Committee.

CDKN2A is the most common high risk susceptibility gene identified to date in familial malignant melanoma. The CDKN2A locus possesses a rather unique genomic organization: it consists of two upstream exons, 1α and 1β, driven by different promoters, that are combined to the common exons 2 and 3 to produce two distinct proteins. The transcript for p16INK4A is initiated from the proximal promoter of exon 1α that is joined to exons 2 and 3, while p14ARF transcript starts from the upstream promoter and is made of exon 1β plus exons 2 and 3 read with a different frame (ARF stands for *A*lternative *R*eading *F*rame). Thus, the CDKN2A particular genome arrangement (Figure 2), likely arisen by gene duplication (Gil & Peters, 2006), and its alternative exon splicing allow to produce two totally distinct proteins from a shared coding sequence (Sherr, 2001).

2.1.1.1 p16INK4A/p14ARF structure and function

More in detail, p16INK4A (p16 for simplicity) belongs to the INK4 protein family, of four members with the capacity to inhibit cell cycle progression; p16 consists of 156 amino acidic residues, spatially organized into four ankirin repeats, which are structural motifs involved in a large number of protein-protein interactions. A single ankyrin repeat is composed by approximately 33 residues that fold into anti-parallel helix-loop-helix structures, linked by β-hairpins (Zhang & Peng, 2000). All these four ankirin repeats are needed for the interaction of p16 with its target: p16 binds to CDK4/6 (*C*yclin-*D*ependent *K*inases) and inhibits the catalytic activity of cyclin D-CDK4/6 complexes, hence maintaining the oncosuppressor pRb in its hypo-phosphorilated state (Lukas et al., 1995), thus preventing G1/S transition.

The p14ARF protein (p14 for simplicity), instead, is made up by 132 residues and there are no recognizable protein motifs in its structure. It can limit aberrant cell proliferation by binding to and blocking MDM2 and, thus, stabilizing p53 (Stott et al., 1998); increased intracellular levels of p53, in turn, mediate cell cycle arrest and apoptosis. Recent evidence, however, support additional roles of p14 in mediating p53-independent responses (Eymin et al., 2003). Although p16 and p14 act on distinct molecular pathways, it is important to point out that both are involved in tumour suppression, despite of species- and cell type-specific differences between the two; moreover, their functions show some overlap, due to functional interconnections between p53 and pRb pathways (Bates et al., 1998; Sherr & McCormick, 2002).

Due to the wide role of CDKN2A in tumour suppression and together with the ubiquitous expression of both p16 and p14 oncosuppressors, one wonders why there is such a specific predisposition to melanoma, and not to other malignancies. This type of "tissue specificity of gene defects" is commonly found in the majority of hereditary tumour syndromes and a

possible explanation relies on how the same gene might possess entirely distinct functions, depending on its cell type specific expression. In the case of p16, in particular, the role of this protein in activating replicative senescence, an irreversible G1 arrest of still metabolically active cells (Campisi, 1997), has been well established. In human melanocytes, p16 is the master regulator of cell senescence (Sviderskaya et al., 2002) that has no other redundant or compensatory pathways in this particular cell type; this might explain why individuals and families with a germline CDKN2A mutation are prone to melanoma development, due to the specific impairment of melanocyte senescence, a crucial barrier against tumorigenesis.

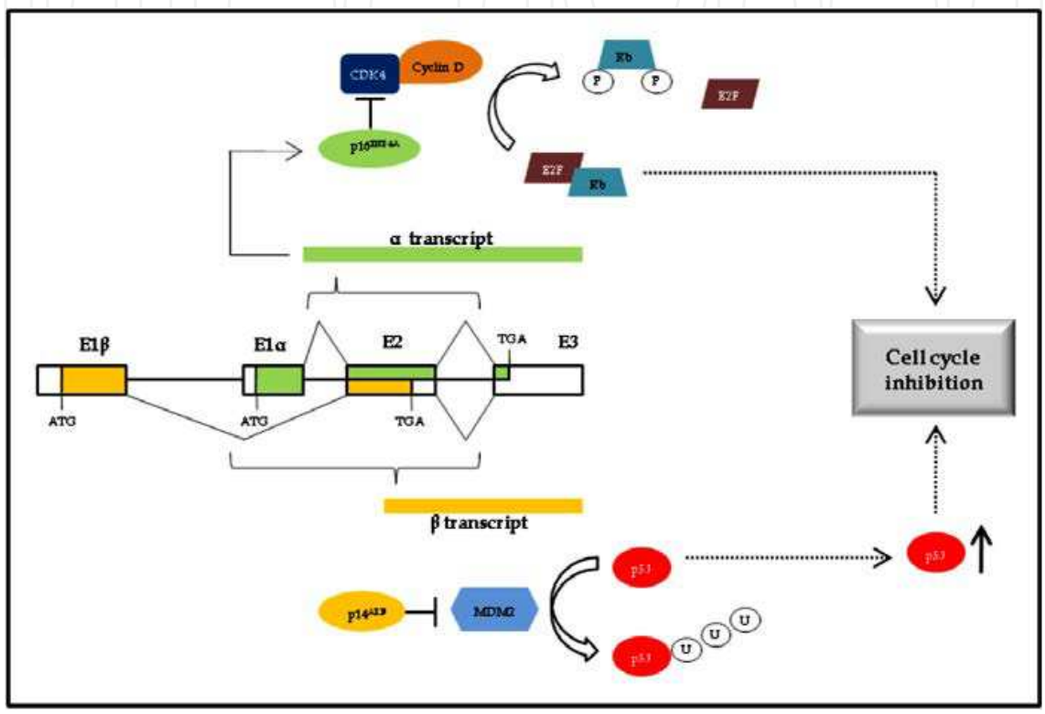


Fig. 2. Genomic organization of CDKN2A locus.

Exons 1α, 2 and 3 code for p16INK4A, able to bind CDK4 and block its activity, thus maintaining the pRb in a hypo-phosphorilated state and, hence, E2F transcriptional factors inactive. Exons 1β, 2 and 3 are joined into the β transcript, coding p14ARF, that inhibits the ubiquitin-ligase activity of MDM2, with a following p53 accumulation. Both, the pRb and the p53 pathways, finally result in G1 cell cycle arrest.

2.1.1.2 CDKN2A and melanoma predisposition

A variety of CDKN2A germline mutations have been identified to date in melanoma-prone families, while they are reported to be rare in the general population. The relative risk of melanoma development conferred by CDKN2A mutations in the general population was estimated by an international case-control study (Berwick et al., 2006). This study determined an overall relative risk of melanoma associated with CDKN2A mutations of 4.3, with considerable variations of this value, depending on the particular type of mutation.
In order to better characterize mutations in this high-risk melanoma susceptibility gene, the International Melanoma Genetics Consortium (GenoMEL), comprising most familial melanoma research groups from North America, Europe, Australia and Middle East,

performed a large study on 446 families with at least three malignant melanoma patients, for a total of about two thousands enrolled patients (Goldstein et al., 2006). Overall, 41% of families had CKDN2A mutations, with differences in the mutation detection rates among several geographic areas. CDKN2A mutations targeting the p14 sequence only (i.e. localized mainly in exon 1β) were much less common and present in only seven families, while mutations affecting the p16 sequence were detected in 178 families and were present in both exon 1α and exon 2. Moreover, the 57 different p16 mutations included: missense mutations (65%), deletions (16%), insertions or duplications (7%), nonsense and splicing mutations (10%). Many of the most recurrent CDKN2A mutations were "founder mutations", originated from a common ancestor and dating back 100 generations (Pollock et al., 1998). Moreover, a significantly younger median age at melanoma diagnosis was observed in mutated families than in families without mutations. Furthermore, the probability to detect a CDKN2A mutation was dependent on the number of melanoma cases within each family (Kefford et al., 1999). Interestingly, the presence of pancreatic cancer was confirmed to be associated with a higher CDKN2A mutation frequency.

Finally, given a certain CDKN2A mutation, what is the risk of melanoma development in a mutation carrier? Again, a study by GenoMEL on 80 families with at least two melanoma cases in first-degree relatives, for a total of about 400 melanoma patients from Europe, Australia and United States, provided data on CDKN2A mutation penetrance (Bishop et al., 2002). Overall, CDKN2A mutation penetrance was estimated to be 30% by age 50 and 67% by age 80, without significant modifications by gender or co-presence of p14 mutations. Importantly, also in this case the penetrance was reported to be strongly influenced by geographic areas and, hence, by environmental exposure: for instance, by age 80 the penetrance was 58% in Europe, 76% in the United States and 91% in Australia, also showing that penetrance varied with melanoma incidence rates among populations.

2.1.1.3 CDKN2A and melanoma/pancreatic cancer syndrome

Approximately 5-10% of pancreatic cancer (PC) cases are attributable to hereditary cancer syndromes (Habbe et al. 2006). Virtually all PCs have somatic inactivation of CDKN2A (Schutte et al., 1997), thus suggesting a critical relevance of this locus in PC pathogenesis and an association between PC and CDKN2A mutations was described. Several authors (Goldstein et al, 1995; Goldstein, 2004; Whelan et al., 1995) showed that PC was found in carriers within melanoma families harbouring CDKN2A mutations and a more recent study showed that individuals possessing the p16-Leiden mutation (a specific 19 base-pair deletion in exon 2) had an estimated cumulative risk of 17% to develop a PC with a mean age at diagnosis of 58 years (Vasen et al., 2000). In 2002, Lynch and co-workers analyzed eight families characterized by melanoma/PC association and presence of CDKN2A mutations, and they proposed the possibility of a new hereditary cancer syndrome, defined as "*F*amilial *A*typical *M*ultiple *M*ole *M*elanoma-*P*ancreatic *C*arcinoma" (FAMMM-PC) syndrome (Lynch et al., 2002).

At present, however, several questions remain, and the molecular mechanisms linking CDKN2A function and PC development need further investigations. Moreover, it is not yet clear which CDKN2A mutations are specifically associated with PC and why.

2.1.2 CDK4

The cyclin-dependent kinase CDK4 interacts with cyclin D and the resulting complexes catalyze the phosphorylation of target molecules that in turn promote the G1/S transition of

cell cycle. As previously discussed, CDK4 activity can be regulated by p16 and mutations in CDK4 gene could perturb the cell cycle control, when they disrupt the ability of CDK4 to bind p16. CDK4 mutations are very rare compared to CDKN2A (Goldstein et al., 2006): they occur in only 2% of families analyzed to date, with a pattern of inheritance and an age of tumour onset similar to that reported for CDKN2A mutations.
The most recurrent mutations are localized in codon 24, replacing an arginine with a cysteine (Zuo et al., 1996) or a histidine (Soufir et al., 1998). These mutations, targeting the p16 binding site of CDK4, render the kinase resistant to p16 inhibition and thus convert CDK4 to a dominant oncogene, with only a single mutated allele required for tumorigenesis.

2.2 Moderate/low risk and modifier genes

2.2.1 More on hereditary genetic risk

High-risk genes alone do not completely account for the heterogeneous genetic substrate underlying familial melanoma. Only a small fraction of melanoma-prone families, in fact, carries mutations in the highly penetrant loci described above. The remaining genetic risk could be due to high penetrance genes not yet identified or, more likely, to other less penetrant, lower risk genes. The so called "polygenic model" states that a large number of alleles, each conferring a small genotypic risk, combine additively or multiplicatively to confer a range of susceptibilities to the population (Houlston & Peto, 2004). Thus, the particular allelic pattern may influence the lifetime risk of an individual, but also expressivity and penetrance of high risk loci, acting as "modifier risk genes". Within low penetrance inheritance, the polygenic component is significantly represented by *S*ingle *N*ucleotide *P*olymorphism*s* (SNPs) that characterize each susceptibility allele. These polymorphisms could directly regulate molecular pathways involved in tumorigenesis initiation and progression (i.e., metabolism dysfunctions, cell death, inflammation, immune response and angiogenesis) or could modulate the host response to environmental factors (DNA-damage repair after sun exposure). The relevance of these numerous low penetrance genes justifies the considerable efforts that were made in their identification, mainly performed by case-control studies on candidate genes and, more recently, by GWAS. So far, several moderate/low penetrance genes were described, but for most of them the clear contribution to cancer development was not yet exhaustively elucidated. Gene functions, redundancy in the cellular pathways and protein pleiotropism represent, in fact, the main complexities in the comprehension of this field (Caporaso, 2002). For familial melanoma, different moderate/low penetrance genes were reported to influence the lifetime risk of developing the disease.

2.2.2 MC1R

The *M*elanoCortin type-*1* *R*eceptor (MC1R) is a seven-pass transmembrane G-protein-coupled receptor, specifically expressed on skin melanocytes. Functionally, MC1R is an upstream component of the intracellular pathway leading to melanin biosynthesis and skin pigmentation. The binding to its ligand α-MSH (*M*elanocyte *S*timulating *H*ormone) activates MC1R, with upregulation of intracellular cAMP levels that, in turn, modulate melanin production (Figure 3). Two melanin types are present in mammals: pheomelanin, a red-yellow pigment, typical of the so-called RHC (*R*ed *H*air *C*olour) phenotype, and eumelanin, the darker pigment that confer olive complexion and brown/black hair. MC1R engagement can shift the intracellular pheomelanin/eumelanin balance, thus increasing the production of the latter and modulating skin sensitivity to sun exposure (Sturm et al., 2001). A

functional impairment in MC1R signalling alters the downstream pigmentation pathways, causing an accumulation of pheomelanin that, in turn, has reduced UV protective capacity and can produce cytotoxic and mutagenic metabolites (Sturm, 1998), accelerating melanocytic transformation. This functional impairment can result from different mechanisms: some variants fail in the stimulation of cAMP production (Beaumont et al., 2007; Garcia-Borron et al., 2005), while others show MC1R reduced affinity for its ligand α-MSH (Ringholm et al., 2004) or there are even defects in desensitization and internalization of the MC1R receptor itself (Sanchez-Laorden et al., 2007).

Given the role of MC1R on epidermal response to UV-induced damage, it is not surprising that the MC1R allelic state influences melanoma risk, by directly regulating skin pigmentation. MC1R locus is highly polymorphic in Caucasians (Savage et al., 2008) and over 100 variants have been identified so far. These include "R" alleles, found to be prevalently associated with light skin, red hair, freckles and sun sensitivity (termed RHC phenotype), all known melanoma risk factors, and "r" alleles, with a weaker or absent association with the RHC phenotype. The molecular basis discriminating between R and r alleles apparently resides in the complete (R) or partial (r) loss of the receptor signalling ability. Recently, it was demonstrated that melanocytes harbouring R alleles possessed markedly reduced surface expression and/or impaired G-protein coupling of the corresponding receptor (Newton et al., 2007).

It is important to underline that MC1R variants have also a different functional causative role on melanoma development and several studies showed that R variants were more strongly responsible for melanoma risk (Kanetsky et al., 2006). In a very recent meta-analysis, the estimated summary relative risk for R and r alleles were found to be of 2.44 and 1.29, respectively, although both estimates were associated with evidence of substantial heterogeneity across studies (Williams et al., 2010). In Italy, some R alleles, such as R151C and R160W, were reported to confer an attributable risk of 7.48 and 4.54, respectively (Raimondi et al., 2008).

By all means, correlations between MC1R allelic status, skin pigmentation features and melanoma onset are more complex. Some "R" variants could not be associated with a RHC phenotype; moreover, MC1R might influence melanoma development by acting on different molecular pathways that are pigmentation-independent such as a MC1R-induced upregulation of p38 MAPK in melanocytes, likely occurring via cAMP (Newton et al., 2007). Potential downstream outcomes of p38 activation include histone H3 phosphorylation with subsequent chromatin remodelling and altered gene regulation, relevant for non-pigmentary pathways including cell cycle regulation, differentiation, and apoptosis.

A variety of studies have reported that MC1R variants can markedly modify the penetrance of CDKN2A locus (Box et al., 2001; Fargnoli et al., 2010; Goldstein et al., 2005, 2007). To this end, a recent study performed by GenoMEL (Demenais et al., 2010) investigated the associations among host phenotype, MC1R variants and melanoma risk in CDKN2A mutation carriers. The analysis, performed on 815 mutation carriers from Europe, North America and Australia, showed interesting results: both non-RHC and RHC variants were associated with increased melanoma risk, that rose threefold in presence of at least one MC1R variant; moreover, the risk was higher in presence of RHC variants and a higher number of MC1R variants. Interestingly, the MC1R-associated risk seemed even greater when CDKN2A mutations affected p16 only, than when they involved both p16 and p14. In addition, hair colour and a high number of nevi are significantly associated with increased melanoma risk; in presence of this host phenotype, the increase in melanoma risk with

anyone of the four more frequent MC1R variants (V60L, V92M, R151C and R160W) remained statistically significant. In conclusion, melanoma risk in CDKN2A mutation carriers is modified by type and number of concurrent MC1R variants, thus confirming the role of MC1R as a risk modifier gene.

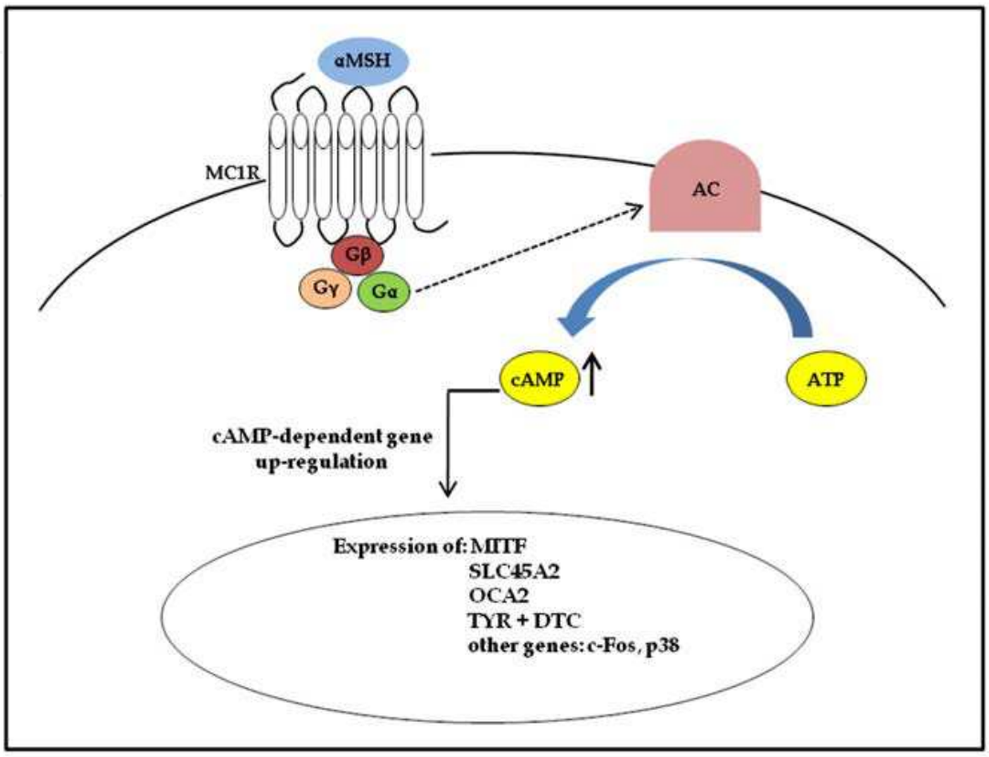


Fig. 3. MSH-MC1R signalling pathway.

The binding of the α-MSH ligand activates G-coupled MC1R, with subsequent cAMP level increase, through the catalytic activity of Adenilate Cyclase (AC). The intracellular cAMP accumulation up-regulates the expression of key genes: MITF plays a pivotal role in mediating transcriptional control of numerous other genes. Activated pigmentary genes include SLC45A2, TYR and DTC, that all modulate melanin synthesis and skin colour, and OCA2, involved in the regulation of the facultative pigmentation. Other MC1R-up-regulated non-pigmentary genes comprise c-Fos, a well-known transcription factor influencing DNA-repair, cell cycle regulation and apoptosis, and p38 MAP kinase (Newton et al., 2007).

2.2.3 Additional pigmentation genes

Human pigmentation is a polygenic trait influenced by a plethora of different genetic determinants. In addition to MC1R, several other pigmentation genes were described to be associated with melanoma risk. A large-scale association study investigated different loci able to influence hair, eye and skin colour (Gudbjartson et al., 2008). Variants at three of these loci showed significant association with melanoma. In particular, the strongest association was found with ASIP locus (on chromosome 20q11.22), coding for *A*gouti *SI*gnalling *P*rotein, a second ligand of MC1R and an antagonist of α-MSH. In principle, mutation in ASIP could mimic a loss-of-function of MC1R; to date, no variants have been

identified in the coding regions of ASIP. The other two associated loci were TYR and TYRP1, involved in pheomelanin/eumelanin biosynthesis.

2.2.4 Other genes

A GWAS carried out by the GenoMEL (Bishop et al., 2009), identified the *M*ethyl*T*hio*A*denosine *P*hosphorylase (MTAP) gene, adiacent to CDKN2A on 9p21, coding the first enzyme of the methionine salvage pathway and having a documented tumour-suppressor activity, as a melanoma-associated locus. MTAP variants were found to be strongly associated with a high melanocytic nevus number (Falchi et al., 2009), one of the strongest known melanoma risk factor.

*E*pidermal *G*rowth *F*actor (EGF) gene was also considered a reasonable risk candidate: a specific SNP, located upstream of the initiation codon of pre-pro-EGF, is more frequent in melanoma patients than in unaffected controls and it is reported to confer a 2.7 relative risk of disease development (Shahbazi et al., 2002).

*G*lutathione *S*-*T*ransferases (GSTs) are a family of isoenzymes largely involved in metabolic processes. Interestingly, GST genes are candidates for modulating CDKN2A penetrance. GST genes *M*u1 (GSTM1) and *T*heta 1 (GSTT1) are specifically expressed in the skin to detoxify products from oxidative stress reactions caused by UV irradiation. A homozygous deletion of GSTM1 (GSTM1 null) is present in about 50% of Caucasians, while about 20% of them carry a homozygous deletion of GSTT1 (GSTT1 null). In particular, combined deletions of both genes were found associated with increased UV sensitivity and UV-inducible skin cancers, and null gene variants were also proposed as melanoma risk factors (Mössner et al., 2007).

Another locus governing metabolic processes and involved in melanoma susceptibility codes for *CY*tochrome *P*450 *D*ebrisoquine Hydroxylase (CYP2D6). Different inactivating polymorphisms were found to be more recurrent in melanoma patients, who were frequently homozygous for these non-functional alleles, than in controls (Strange et al., 1999).

Additionally, it was shown that a SNP in the MDM2 gene, the SNP309 (T>G variation) was linked to the tumour onset and outcome. However, discordant results were reported on the effect of this SNP on age at diagnosis of cutaneous melanoma in Caucasian female population and no associations were found among SNP309, melanoma risk, age at diagnosis and presence of metastasis in the Italian population, although SNP309 was significantly associated with tumour Breslow thickness (Capasso et al., 2010).

It was proposed that the *V*itamin *D R*eceptor (VDR) gene might play a role in melanoma development as well, since its interaction with the calcitriol ligand results in antiproliferative and pro-differentiation signals on melanocytes. Rare VDR alleles were found more commonly in melanoma cases than controls, although conflicting evidence were also reported (Barroso et al., 2008; Hutchinson et al., 2000).

All these evidence stress the fact that melanoma, being a multifactorial disease, is characterized by a heterogeneous genetic background and further investigations on melanoma-predisposing genes are necessary to gain new insights into this overwhelming wealth of information and, in turn, to the comprehension of melanoma pathogenesis.

3. Genetic counseling and testing for familial melanoma

3.1 Genetic screening: how and why

In general terms, screening is a systematic attempt to identify, among apparently healthy individuals, those at higher risk for a specific disease, to finally inform them about

preventive protocols. The aim of cancer screening can be either to identify precancerous lesions, whose treatment will be truly preventive (primary prevention), or to diagnose cancer at an earlier stage and treat the tumour more efficiently (secondary prevention).
In the case of cancer syndromes, the existence of inherited alterations in tumour-predisposing genes can be detected by a genetic test, which is a DNA analysis to determine the presence of a mutation that could be responsible for the development of the disease. Thus, for hereditary cancers, genetic testing is used to predict the risk of a future health impairment (Tsao & Niendorf, 2004).
The application of each genetic test in the clinical practice should be considered in conjunction with a formal and qualified genetic counseling setting, in which an inter-disciplinary team of specialists, such as geneticists, clinicians and psychologists, provides a comprehensive consultation service, with the purpose to elicit the patient's personal and familial health history, to assess genetic risk, to discuss with patients and families about benefits, limitations, interpretations and possible implications of genetic testing and diagnosis, to assess the need of a psychological support, and to include individuals in appropriate screening programs (Niendorf & Tsao, 2006).
The methodology of genetic counseling implies, firstly, the collection of the family pedigree, which is a genealogic tree used to analyze the mendelian inheritance of a certain trait, such as the presence of a mutation in a cancer-predisposing gene. It is particularly important to include the widest amount of information about the number and type of cancer-affected patients, their age at onset, the presence of multiple cancers in the same individual and the occurrence of cancers known to be associated with the syndrome of interest (as in the association of pancreatic cancer with familial melanoma). All the collected data allow prediction of the so called "theoretical risk", given as the product between the probability to have inherited a mutation in a susceptibility gene and the mutation penetrance at a given age. In general, the presence of two or more affected first-degree relatives, an early age at onset and a personal history of multiple primary cancers are features that strongly suggest an inheritable genetic cancer predisposition. In such a case, a genetic test for the molecular characterization of a given cancer-predisposing gene can be offered to the proband, the first affected member of the family enrolled for the screening, in association with an informed consent about the predictive power and the limits of the test.

3.2 Genetic test for CDKN2A

The aim of genetic testing for CDKN2A is to identify which family members are mutation-carriers, in order to target these higher risk individuals to more intensive cancer prevention and surveillance.
In a recent study, the predictive value of personal and familial history of melanoma/ pancreatic cancer was assessed for the identification of individuals at increased probability to harbour a CDKN2A mutation (Leachman et al., 2009). The study demonstrated that in higher melanoma incidence areas, individuals with multiple primary melanomas and/or families with at least one invasive melanoma and two or more melanoma and/or pancreatic cancer cases among first- or second-degree relatives in the same familial side represented appropriate candidates for genetic evaluation. On the other hand, in geographic areas with lower melanoma incidence rates, two melanoma and/or pancreatic cancer cases within a family could be sufficient and appropriate for a genetic investigation.

When performed, genetic testing is based on the mutational analysis of CDKN2A locus: genomic DNA, extracted from *P*eripheral *B*lood *M*ononuclear *C*ells (PBMCs) of the patient, is subjected to sequencing techniques to analyse the whole gene sequence, including exonic and intronic regions, splicing junctions and 3'/5'UTR. A written informed consent has to be obtained for all tested patients under local ethic committee-approved protocols.
The interpretation of the possible results of a genetic test represents one of the most delicate issues in this context (Udayakumar & Tsao, 2009) and must be done by specialists with genetics expertise. When a specific mutation is detected, the patient might develop a melanoma during life, because of his increased risk, compared to the general population, due to the occurrence of the inherited mutation. The detected mutation provides a sort of familial genetic signature and other family members could be tested for it (specific test). When the mutation occurs within the family, but it is absent in a given member (non-carrier), the result of the specific test is negative. However, even in presence of a negative specific test, an increased risk for family members still persists, due to the possible co-inheritance of other shared genetic, risk-modifier factors. On the other hand, and most often, when no mutation is detected in a melanoma-prone family, the test does not provide any new information; in this case, other types of CDKN2A mutations or other melanoma-predisposing genes could be involved in determining that specific pedigree, hampering the possibility to rule out hereditary melanoma. Of course, families comprising individuals with uninformative tests are considered at increased risk, irrespective of their DNA status. An uninformative test is also obtained when a genetic alteration with unknown functional significance (the so called "*U*nclassified *V*ariants", UVs) is identified, as later discussed in more detail.
For all these reasons, it is generally recommended that members of melanoma families should be invited to participate in screening and cancer prevention programs, regardless of their CDKN2A mutational status (Hansson, 2008; Kefford et al., 1999). Primary prevention measures mainly include education for sun protection and routine skin self-examinations, while secondary prevention is focused on monitoring pigmented skin lesions, that could be considered as melanoma precursors, since early detection and surgical excision are the only powerful means presently available to improve melanoma prognosis.
Undoubtedly, controversies on genetic tests for melanoma still exist (Kefford et al., 2002; Kefford & Mann, 2003), but the clinical utility of CDKN2A testing continues to improve and its potential benefits have been already established.

3.3 Clinical counseling in Italy

The variable melanoma incidence and CDKN2A mutation penetrance among countries render unfeasible the definition of international guidelines to regulate the access to genetic testing for CDKN2A, insomuch as specific selection criteria must be applied on a national scale. In Italy, one of the first countries where genetic testing for familial melanoma was offered in medical or genetics services, the Italian Society of Human Genetics (SIGU) outlined recommendations on genetic counseling and testing for familial melanoma, suggesting to offer genetic test for CDKN2A to those families with at least two first-degree affected members. A recent cooperative study, among 9 Italian centres, quantified the frequency of CDKN2A mutations in melanoma-prone families, in line with the SIGU eligibility criteria for clinical counseling, as summarized in Figure 4 (Bruno et al., 2009). This analysis, of 208 Italian families that met the SIGU criteria and underwent genetic testing, reported that 33% of the families overall carried CDKN2A mutations. More in detail, the results showed that CDKN2A mutation frequency rose with the number of affected

members in the family, that the median age at diagnosis was significantly different in individuals from CDKN2A-mutated families (42 years), compared to patients from families without mutations (49 years), and that the CDKN2A mutation frequency increased with the number of patients presenting multiple primary melanomas within the family. In particular, families with two cases accounted for 71% of the entire sample and for 52% of the families harbouring CDKN2A mutations. These findings suggested that if stricter selection criteria for genetic test were applied in Italy (such as the presence of at least three affected members), a significant subset of CDKN2A mutated families would not be identified, a result that outlines how the definition of selection criteria to access to genetic testing should depend upon the particular geographic area of interest.

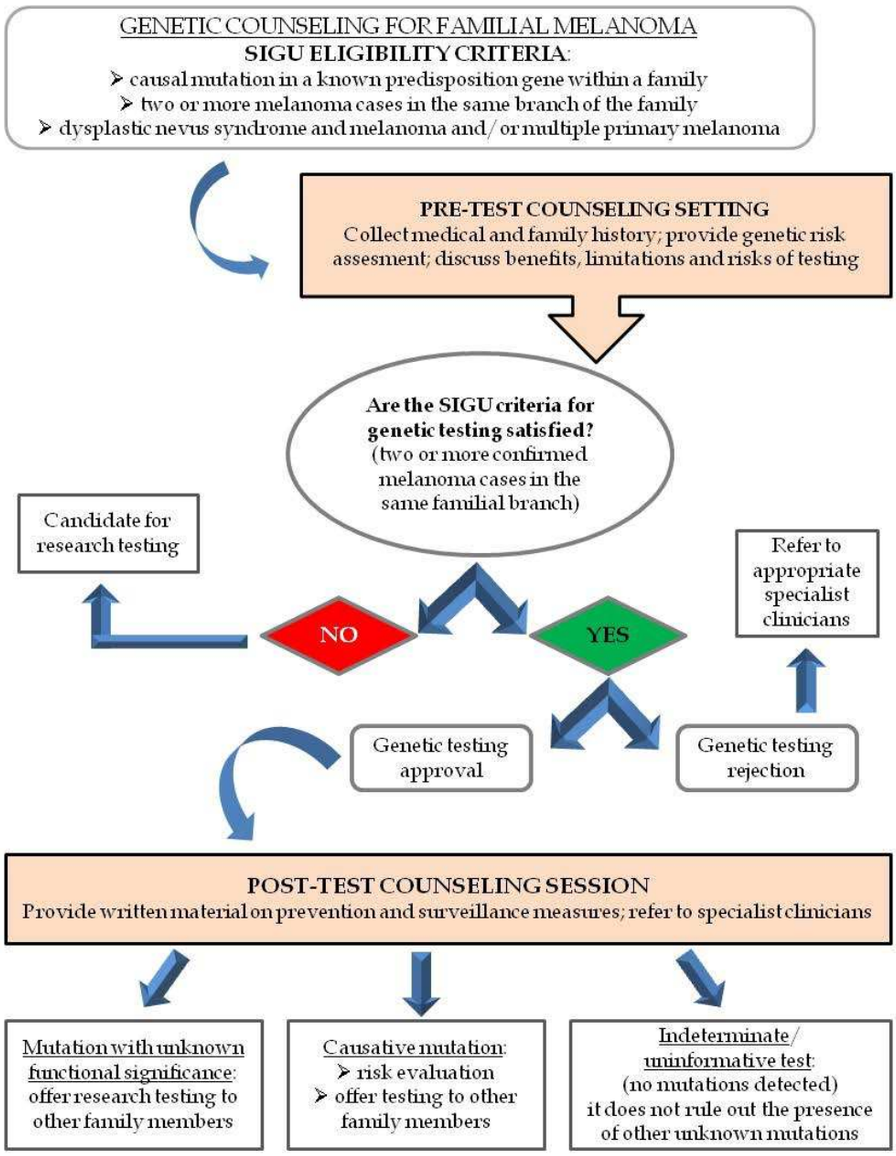


Fig. 4. Italian Society for Human Genetics (SIGU) recommendations for clinical genetic counseling and testing for familial melanoma (Bruno et al., 2009, with modifications).

4. The problem of CDKN2A unclassified variants

As previously anticipated, a possible outcome of genetic testing for CDKN2A can be the identification of an alteration with undetermined pathogenetic significance, that is an *U*nclassified *V*ariant (UV), a result that can complicate rather than improve cancer risk assessment. In this case, a change in the nucleotide sequence is found, but there is not enough information to decide whether it affects or not the function of the gene product and, thus, cancer predisposition. UVs are mainly represented by non-synonymous changes in a given protein position, also called "missense variants". Over 150 different UVs of CDKN2A, mostly targeting p16, were reported worldwide (Goldstein et al., 2004, 2006). The main features of these UVs were grouped together and are now also available on the *L*eiden *O*pen *V*ariation *D*atabase (LOVD; http://chromium.liacs.nl/lovd2/home.php?select_db=CDKN2A).

Obviously, an uninformative result can be a source of anxiety for individuals and their offspring because they will not be able to use this information for clinical purposes. In addition, all first-degree relatives, including non carriers, are considered at risk as long as the contribution of the variant to disease cannot be assessed, resulting in frequently unnecessary psychological stress and lifelong screening. The distinction between a true pathogenic mutation and a benign variant represents an essential prerequisite to univocally distinguish individuals at higher risk and, consequently, to enroll them in prevention protocols. In the last few years, a growing number of sequence changes of undetermined clinical significance was reported, and this issue received the attention of a large number of investigators. Recently, the *I*nternational *A*gency for *R*esearch on *C*ancer (IARC; the cancer research branch of the World Health Organization) convened a Working Group on UVs in high-risk cancer susceptibility genes, CDKN2A included. The Group comprised investigators of different specialties with the aim to establish a standard approach to UV evaluation. Discussions and specific recommendations of the Working Group were reported in a series of articles published in the November, 2008 special issue of *Human Mutation* (Tatvigian et al., 2008a).

Currently, there is a general agreement for UVs classification on the basis of a multicomponent model, integrating direct and indirect evidence, including genetic, bioinformatic and experimental data, thus providing the highest degree of accuracy in UVs assessment.

4.1 Direct evidence of pathogenicity

Various lines of evidence were proposed to address UV evaluation (Goldgar et al., 2004, 2008). Some types of evidence determine a more direct association between presence of the variant and cancer development. The most straightforward genetic evidence is the co-segregation of a given variant with the disease in pedigrees. It offers the advantage to depend only on the availability of DNA samples from several individuals belonging to families with the variant, although its power is limited by the number of informative meiosis, very low in small pedigrees, and of affected members in the family. A second direct evidence is the evaluation of the variant frequency in case-control studies, particularly suitable for common genetic variants. For most UVs, however, their rare frequency would require prohibitively wide sample sizes to demonstrate its pathogenicity. Practically, this method is mainly used to rapidly screen out potentially neutral variants.

An additional evidence relies on co-occurrence analysis: in principle, if there is a known pathogenic mutation in a melanoma family, its presence will decrease the probability that a

given variant, found in association with that mutation, could be pathogenic as well. In other terms, a probably pathogenic variant will not be found in co-occurrence with a *bona-fide* mutation.
In the case of CDKN2A, the frequency of rare variants, the low number of carriers within melanoma families and the small pedigrees make difficult the application of all the above mentioned evidences.

4.2 Indirect evidence of pathogenicity: *in silico* analysis

Recently, computational approaches, predicting potential effects of a missense variant on protein structure/function and activity, were developed and widely used to support the classification of UVs (Tavtigian, 2008b). Different computational tools are based on the pairwise comparison of the physico-chemical characteristics or evolutionary substitution frequencies between the wild-type and variant amino acid. The basic principle is that in almost all proteins some amino acid positions, critically relevant for protein function, are highly philogenetically conserved, and in general disease-associated variants are preferentially localized in these positions. Thus, the evolutionary conservation of the amino acidic position, at which the variant occurs, provides an indication of its putative pathogenicity. In addition to the conservation degree, the comparison of the physico-chemical characteristics between any wild-type and variant amino acid position is taken into account, in order to evaluate the biochemical severity of the substitutions. Additional tools, based on similar theoretical assumptions, operate in *P*rotein *M*ultiple *S*equence *A*lignments (PMSAs) across different species. Likewise, missense substitutions falling at gene positions that are evolutionary constrained are more likely predicted to be pathogenic.
Known examples of methods based on evolutionary fitness and/or on biochemical parameters are represented by PAM 250 and BLOSUM 62. They are amino acid substitution scoring matrices derived from the frequencies with which the 20 amino acids are observed substituting each other in PMSAs of related proteins (Henikoff & Henikoff, 1992); they assign to each variant a different score value, depending on its substitution frequency, that eventually defines the likelihood to be deleterious, rather than neutral.
Another missense substitution analysis algorithm is the Align-GVGD, based on the Grantham difference, that describes the difference in side-chain atomic composition, polarity and volume between any two amino acids (Grantham, 1974). Again, a score is attributed to the variant, according to the "conservative", "non-conservative", or "radical" substitution features.
More complex algorithms consider also protein structural properties, generally available when the three-dimensional crystal protein structure has been solved. The structural features provide information about the possible location of the variant into binding sites or enzymatic active sites, secondary structural motifs (helices, sheets, loops, etc.) or about its solvent accessibility. In these terms, an amino acid substitution that presumably perturbs or disrupts such structural conditions is predicted to be deleterious for the protein function.
Example of algorithms, based on a combination of biochemical parameters, PMSAs and structural features, include PMut (Ferrer-Costa et al., 2005), SIFT (Ng & Henikoff, 2003) and PolyPhen (Sunyaev et al., 2001).
Due to their ability to analyze the expected effects of each individual variant, they were applied to the analysis of UVs of different cancer-predisposing genes, including CDKN2A (Chan et al., 2007). A recent study on a series of CDKN2A missense variants, however, showed potential limits of these approaches: widely conflicting results, in fact, were

obtained, with several specific variants, that were paradoxically predicted to be benign or pathogenic, depending on the software used (Kannengiesser et al., 2009).
Undoubtedly, *in silico* approaches provide an excellent support for UVs classification, but their accuracy is apparently limited by the available evolutionary, mutational or structural databases and, in some cases, by intrinsic limitations of each individual method. Therefore, *in silico* analysis represents an ancillary strategy for the evaluation of UV pathogenicity, but it needs to be validated and complemented by additional, stronger evidence.

4.3 Indirect evidence of pathogenicity: Functional analysis

Indirect UV evaluation can be also performed by using laboratory tests that can measure the effects of a variant on the activity of the gene product (Couch et al., 2008; Olilla et al., 2008). The rationale for the use of functional assays relies on the fact that the detection of a decrease in activity of a tumour suppressor gene, due to the constitutive presence of a variant in its sequence, likely results in an increased cancer predisposition. Thus, functional assays able to quantify a reduced or altered protein function can be employed to potentially predict the outcome of the UV on protein activity and function.
A powerful assay must be designed according to the functional properties of the encoded protein and hence each single cancer-predisposing gene requires the development of a set of specific tests.
A variety of p16 missense variants were investigated by functional analysis (Kannengiesser et al., 2009; McKenzie et al., 2010; Ruas et al., 1999), since two main p16 functions can be easily measured in vitro: the CDK4/6 binding capacity and the p16 ability to arrest cell-cycle. In particular, the advantage of using cell growth inhibition assays is that they evaluate a phenotype directly involved in tumorigenesis. Other functional tests developed for p16 are discussed in the following paragraph.
Undoubtedly, several unsolved issues on the applicability of functional tests still remain. Firstly, the results obtained by various assays often disclose a relevant discordance, with the same variant showing a different behaviour, depending on the assay used. Approximately, one-half of the variants tested so far in literature possess normal CDK4/6 binding, but fail to trigger cell-cycle arrest. This indicates that a single assay is not sufficient to draw definitive conclusions, and multiple assays must be performed. To this end, a general consensus on the definition of the most suitable panel of assays to be used is still lacking. An additional complication for interpretation of test results relies on the fact that the variants show a continuum of impaired activity, ranging from UVs similar to the wild-type function to UVs with a complete loss-of-function. It is therefore necessary to establish threshold and cut-off values, for eventually distinguishing UVs with wild-type, intermediate or loss-of-function behaviours. Again, a general consensus on the degree of functional loss, which is required to classify a UV as pathogenic, is still missing. Nevertheless, there are no doubts that functional tests can provide significant information about the *in vitro* activity of an UV.

4.3.1 Functional approach for a CDKN2A coding UV

Recently, an exhaustive analysis on a p16 missense variant, as an example on the use of functional methods, was performed by our group (Scaini et al., 2009). The Gly23Asp (G23D) was identified in a family with three melanoma cases, even if only one of the two tested patients was a carrier; the same variant was previously reported in a French and in another Italian melanoma family. The aa protein position 23 falls within the ankyrin consensus sequence and other variants at codon 23 were reported, with different evidence indicating

some involvement in melanoma predisposition. Co-segregation of G23D with the disease was observed only in one of these three families, thus rendering this analysis to be not conclusive for the assessment of the variant pathogenicity. The presence of phenocopies, furthermore, might be likely due to the relevant role played by low penetrance genes, acting together with environmental factors within a family. Hence, the application of indirect lines of evidence, namely functional assays measuring key cellular p16 functions, was particularly useful for this variant classification. The protein ability to arrest cell cycle was evaluated by i) proliferation curves, showing the trend of cell growth in time, ii) colony efficiency assays, testing colony formation capacity when cells were seeded at low density, and iii) flow cytometric analysis, indicating a decreased percentage of G1-arrested cells, compared to p16-wild-type ones. Additionally, immunoprecipitation and mammalian two-hybrid binding assays were employed to measure p16 ability to bind CDK4, while pRb phosphorilation was analysed by Western blotting. Furthermore, we confirmed previous evidence that some p16 deleterious variants show an altered cellular localization by immunostaining followed by fluorescence microscopy, forming both cytoplasmic and nuclear aggregates, possibly due to an incorrect folding of the mutant protein during post-translational processing. The results obtained from all these functional tests, summarized in Figure 5, clearly showed an important impairment in G23D function, compared to p16 wild-type. The experimental results were also found to agree with *in silico* predictions, thus supplying sufficient evidence to classify the variant as a "loss-of-function" mutation, which most likely predisposes carriers to melanoma development.

4.3.2 Beyond missense variants: CDKN2A non-coding UVs

Together with missense UVs, germline polymorphisms outside the coding regions of CDKN2A (i.e., promoter, splicing sites, 5'UTR and 3'UTR) were also detected (Hayward, 2000). The first defined pathogenic mutation was the 5'UTR -34G>T transversion, which gives rise to an alternative translation initiating codon with a decreased usage of the wild-type AUG, likely derived from a common founder in the United Kingdom (Liu et al., 1999). Two extensive studies screened more than 1kb of the p16 untranslated and promoter regions in search of mutations in English, Italian, American (Harland et al., 2000) and Australian families (Pollock et al., 2001); polymorphisms at positions -33, -191, -493, and -735, as well as three novel variants at positions -252, -347, and -981 were identified. However, these novel variants did not segregate with disease and were, thus, classified as rare polymorphisms. Rare polymorphisms or variants at the CDKN2A 5'UTR are currently defined as UVs after determining their frequency in control subjects and following co-segregation analysis, when possible. While several functional tests for determining the pathogenicity of missense germline mutations in the coding regions were developed, there are no studies addressing the possible impact of promoter/5'UTR variants on p16 transcription/translation, except for a single publication by an Italian group (Bisio et al., 2010). Reporter assays were developed to study a panel of p16 5'UTR variants, recently identified in a hospital-based series of melanoma cases selected within an ongoing case-control study from an Italian population. Polysomal profiling was also applied as a means to determine the relative impact of the 5'UTR variants on mRNA translation efficiency in heterozygous patient cells. Overall, the results provide tools to assess the functional significance of non-coding 5'UTR mutations and strongly suggest that the -21C>T non-coding variant can be of clinical significance for melanoma predisposition due to its negative impact on the post-transcriptional dynamics of p16 mRNA.

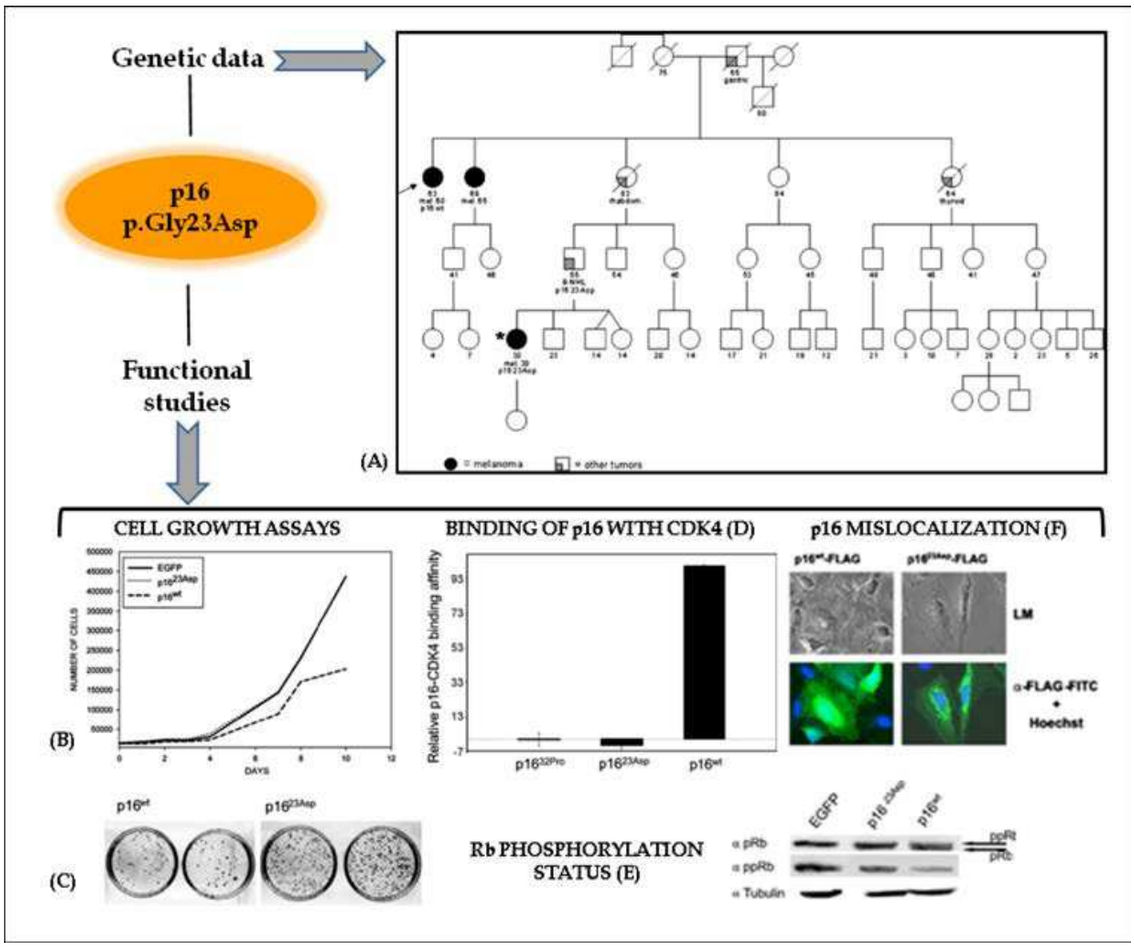


Fig. 5. Functional analysis of the G23D missense variant (Scaini et al., 2009). (A) Pedigree of the Italian family harbouring the variant. Mutational analysis of the first proband (indicated by arrow) was uninformative, while the patient marked with asterisk and her father carried the variant. (B) Proliferation curve and (C) Colony efficiency assays, showing the aberrant cell growth of $p16^{23Asp}$ transfected cells. (D) Impressive reduction in CDK4 binding of the variant, compared to wild-type p16. (E) Analysis of pRb phosphorilation status showed a variant-associated accumulation of hyper-phosphorilated pRb. (F) Mislocalization analysis revealed presence of cytoplasmic and/or nuclear $p16^{23Asp}$ aggregates. All functional tests were performed in U2-OS or NM-39 cell lines.

5. Mutational analysis of melanoma-predisposing genes in Italy

As previously discussed, Italy is considered a low melanoma incidence country and Italian melanoma-prone families are generally characterized by a small number of cases (mostly two affected relatives within the same family).

The estimated cumulative risk of developing melanoma over a lifetime in the Italian population is 0.5% (Balzi et al., 1997), unlike in the United States, Australia, and New Zealand, where the risks are 2.0% (Jemal et al., 2008), 3.3% (Holland et al., 1999), and 5.7% (Jones et al., 1999), respectively.

Because of this low melanoma incidence, a familial clustering in a Mediterranean country like Italy is particularly indicative of an inherited predisposition, since it seems very unlikely that such a familial aggregation could be due to chance alone. So, investigations addressing the involvement of this genetic component and the characterization of melanoma-associated gene alterations represent a relevant aspect of familial melanoma research in Italy.

Recently, a comprehensive study, reporting data on CDKN2A/CDK4 mutational analysis, was published (Bruno et al., 2009; see section 3.3). The power of this study relies on some significant characteristics: i) it merges melanoma families and patients recruited by nine different centres spread from Northern to Southern Italy, all enrolled under the same eligibility criteria; ii) it quantifies mutation frequency values on a national scale; iii) it defines how mutation frequencies relates to other familial melanoma features, including the number of affected members in the family, the age at onset, and the presence of multiple melanomas. This study estimated a CDKN2A mutation rate of 33% overall, but single studies on families from different Italian regions reported variable mutation frequencies, with high values (>35%) for Liguria (Ghiorzo et al., 1999; Mantelli et al., 2002) and low values (7.3%) for Emilia Romagna and Marche (Landi et al., 2004). Moreover, the mutation frequency in melanoma families is approximately 17-22% for Lazio and Toscana in Central Italy (Binni et al., 2010; Gensini et al., 2007). Importantly, the frequency was found to be higher in regions with founder mutations (i.e. Liguria and Toscana, as later discussed).

Further data on the genetics of familial melanoma in Italy come from a variety of different studies, heterogeneous in methodology and performed on narrow and localized regions. For this reason, each study adds relative results that need to be merged together for a better description of the mutational spectrum of familial melanoma in Italy.

5.1 Italian studies on high penetrance melanoma genes

Different CDKN2A germline mutations have been identified in Italy in the last two decades. As reported worldwide in other mutational studies, missense mutations are the predominant part of CDKN2A alterations and are scattered along its entire coding region. Nonsense mutations, splicing/intronic and regulatory mutations, as well as insertions/deletions, have been described with a much lower prevalence.

5.1.1 Founder mutations

While some mutations were observed only once, others were repeatedly found among families. The most recurrent CDKN2A missense mutation, in Italy and worldwide, is the Gly101Trp (G101W), identified in various families from different countries and particularly prevalent in French and Italian populations. Given its high frequency, the G101W was largely investigated, and its genetic origin was assessed using several families from Italy, France and United States (Ciotti et al., 2000). The haplotype analysis, carried out by means of eight polymorphic markers spanning the CDKN2A locus, revealed no evidence for mutational heterogeneity and suggested that the genotyped families derived from a single ancestral haplotype on which the mutation firstly occurred. The authors finally stated that although it was not possible to unequivocally determine the precise geographic location where the mutation arose and how it spread around the world, it is likely that the mutation originated in South-western Europe. Subsequently, the common genetic origin of the G101W was confirmed by a French study (Auroy et al., 2001). Being a so common mutation, it gained the attention of other researchers and was also functionally characterized.

Interestingly, although the G101W showed some residual binding to CDK4/6 (McKenzie et al., 2010; Parry & Gordon, 1996), it was found to be clearly impaired in the ability to block cell cycle progression (Kannengiesser et al., 2009). These singular results obtained for the G101W emblematically represent the case of a mutation having discordant functional behaviours, on the basis of the particular assay used, as discussed before.
Another frequent founder mutation identified in Italy was the Glu27X (E27X) mutation (Ghiorzo et al., 2006). In particular the E27X, co-segregating with the disease, was found in patients living in, or originally from, a very small area on the North border of Liguria, in North-western Italy. The mutation, located in exon 1α, determines a premature termination codon with a mutant RNA transcript containing only 27 codifying codons, which results in the synthesis of a truncated protein, and possibly in p16 haploinsufficiency. Interestingly, the E27X is the first stop codon CDKN2A founder mutation detected in melanoma families presenting also PC and neuroblastoma, while PC was preferentially reported in association with exon 2 mutations, impairing both p16 and p14.
In a study addressing the frequency and spectrum of CDKN2A/CDK4 mutations in families from central Italy, a third Italian founder mutation, the Gly23Ser (G23S), was detected (Gensini et al., 2007). Again, the haplotype analysis revealed a single common origin for the G23S and several lines of evidence (co-segregation with the disease and case-control studies) suggested its pathogenicity and its involvement in melanoma predisposition.
Taken together, these data show that the major burden of CDKN2A-associated familial melanoma in Italy can be attributed to a limited number of mutations which spread nationwide through founder effects.

5.1.2 Other missense mutations

More than ten years ago, when evidence for CDKN2A involvement in familial melanoma seemed still controversial, a European collaborative work headed by Fargnoli and coll (1998) was performed, to better establish the role of CDKN2A in melanoma predisposition. Four independent missense mutations in exon 1α and exon 2 were detected in four of ten tested families, while no mutations in exon 1β were found. The Gly23Asp (G23D) and the Asn71Ile (N71I) variants, showing co-segregation with the disease, were both located within consensus amino acid residues of the ankyrin repeats, crucially involved in p16 function. The Arg24Pro (R24P) and the Pro114Leu (P114L) were located in exon 1α and exon 2, respectively, and for the former a defective binding with CDK4 was previously reported (Harland et al., 1997).
Another Italian CDKN2A mutational analysis revealed a novel mutation, the Pro48Thr (P48T), showing co-segregation with the disease (Della Torre et al., 2001). The proband carrying the P48T mutation was a woman who developed 4 primary melanomas before age 56; moreover, she belonged to a large family, presenting single and multiple melanomas, oral and colon cancers, bone tumours and other additional cancer types. In the same study the P48T was functionally characterized and it was indistinguishable from wild-type p16 in its ability to interact with CDK4/6, but it had a reduced capacity to inhibit cell growth with a defect in G1/S arrest clearly detectable by flow cytometry.
Another study investigating the relations between CDKN2A mutations and single/multiple primary melanomas in North-western Italy, reported additional missense mutations detected for the first time in an Italian population and the novel Thr77Ala (T77A), never described before (Pastorino et al., 2008).

CDKN2A mutations were also analyzed in 55 families mainly from Emilia Romagna and Marche, in North-central Italy (Landi et al., 2004). A novel mutation, the Leu65Pro (L65P), was identified. Structural considerations on p16 tertiary structures suggested that the variant could disrupt secondary structures, possibly resulting in a small spatial distortion. The PolyPhen calculations failed to predict an impact of the mutation on protein function. Therefore, a yeast two hybrid system was employed and a decrease of about 50% in the binding of L65P to CDK4, compared to the wild-type p16 was observed. Being the leucine at position 65 not conserved across species and considering that molecular modelling suggested only a little effect on this amino acid substitution on protein structure, the modest reduction in CDK4 binding of L65P is very reasonable.
Obviously, not every CDKN2A missense variant necessarily predisposes to melanoma or exerts a pathogenic effect on protein function. In this regard, it was suggested that the Ala148Thr (A148T) variant, located in the fourth ankyrin repeat domain of p16, could be a low penetrance melanoma predisposing allele in a Polish population. To determine the role of the A148T on melanoma risk, this allele was genotyped in French and Italian population (Spica et al., 2006). Although discordant data were previously reported in different series of melanoma patients, the study showed no association of the A148T and melanoma risk.
Other CDKN2A missense mutations have been identified in Italy, although at low frequencies and are reported in Figure 6.

5.1.3 Mutations in p14

Mutations in p14 are less frequent compared to those involving p16, also in Italy. While mutations laying in exon 1β specifically target p14 only, mutations occurring in the shared exon 2 of CDKN2A can potentially affect both p16 and p14, as exemplified by the case of the Asn71Ile (N71I) missense mutation, already discussed (Fargnoli et al., 1998). The N71I is originated by the 212 A>T nucleotide substitution in exon 2, that converts an asparagine to an isoleucine, within the p16 reading frame. The A>T base change also modifies the coding region of the p14 transcript, causing the substitution of a gluthamine with a histidine.
In a case-case study matching amelanotic and pigmented melanoma, the p14 g.193+1 G>A germline mutation was detected for the first time in Italy, in a family with five melanoma cases and a neural system tumour (Ghiorzo et al., 2009). The g.193+1 G>A was previously described to occur in a mutation hotspot at the p14ARF splice site and to be associated with aberrant splicing (Harland et al., 2005). By a research group operating in Central Italy, and studying 155 either familial or sporadic multiple primary melanoma cases, p14 mutations were identified in three unrelated melanoma pedigrees, and no mutations were found in sporadic patients (Binni et al., 2010). Two of these families harboured the g.193 + 1 G>A mutation, while the third family was positive for the g.161 G>A variant, that resulted in the p.Arg54His (R54H) amino acid change. The R54H clearly showed co-segregation with the disease and was classified as deleterious by *in silico* analysis, since the substituted residue is highly conserved among species.
CDKN2A missense mutations detected in Italian melanoma-prone families are listed with their main features in Table 1.

5.1.4 CDKN2A rearrangements analysis

Given that point mutations have a relatively low frequency in familial melanoma, it is possible that other types of CDKN2A alterations, not detectable by routine PCR-based methods, might be involved in a fraction of melanoma cases.

To assess the role of CDKN2A large, quantitative alterations in the Italian population, 124 melanoma families without detectable CDKN2A or CDK4 point mutations were screened by *M*ultiplex *L*igation-dependent *P*robe *A*mplification (MLPA) and real-time quantitative PCR (Vignoli et al., 2008). This study, involving different Italian centres, reported that no gross rearrangements in the CDKN2A coding regions and in the p16-specific promoter were present. In five samples a 6 bp deletion was found in proximity of the promoter region of exon 1β, but further investigations likely indicated it as a low frequency polymorphism, not implicated in disease predisposition.
Similar results were obtained by Binni et al. (2010) on additional melanoma kindreds, thus confirming that CDKN2A rearrangements are an infrequent mechanism for melanoma predisposition in Italy, in agreement with data described in other European countries, reporting that only 2.1% of mutation carriers harboured large CDKN2A rearrangements (Lesueur et al., 2008).

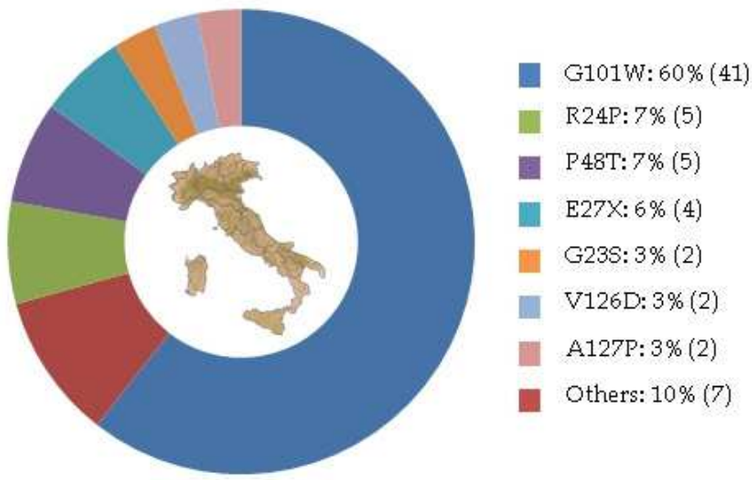


Fig. 6. CDKN2A missense mutation pattern in 208 Italian melanoma families.

An overall mutation frequency of 33% was determined. The frequency at which the single mutations occurred in positive-families is shown, with the number of carrier families in brackets (data extracted from Bruno et al., 2009).

5.1.5 CDK4 mutations

As mentioned before, CKD4 mutations are extremely rare: a CDK4 missense mutation in exon 7, converting the arginine 240 in glutamine (Arg240Gln), was identified (Landi et al., 2004). The mutation was found in the proband and also in two unaffected relatives. Furthermore, in a molecular analysis on subjects with familial and/or multiple primary melanoma, the Arg24His was detected in a family, presenting several melanoma cases (Majore et al., 2008).

5.2 Contribution of low penetrance genes and/or other risk factors to melanoma susceptibility in Italy

As already mentioned, a familial aggregation in a Mediterranean country like Italy is suggestive of an inherited predisposition (Calista et al., 2000). Moreover, melanoma in a

population with a wide range of pigmentary phenotypes, small sized nevi, and intense sun exposure may reveal susceptibility pathways specific for this population. Since no evidence of linkage to other loci for melanoma susceptibility in Italian CDKN2A-negative families was reported (Kerstann et al., 2008; Landi et al., 2004), it is likely that, in some Italian regions, clustering of cutaneous malignant melanoma cases might also be the result of a combination of multiple low penetrance alleles and/or shared sun exposure habits. The MC1R gene is a major determinant of skin phototype and pigmentary characteristics, such as skin, hair and eye colour, that together with exposure to environmental ultraviolet radiation, are the main modulators of individual melanoma risk. Pigmentation and, consequently, sun sensitivity are polygenic traits and several variant alleles have been identified in different regulatory genes (Duffy et al., 2010; Fernandez et al., 2009). Up to now, a national Italian work summarizing the impact of these genes and environmental/physiological factors on melanoma risk is still lacking, while a puzzle of information coming from several Italian regions is mounting, giving only a partial description of the various contributing factors. On the other hand, despite lacking a view of the whole Italian situation, several authors suggest that this might be the right way forward for a correct estimate of low penetrance gene effects, since mutation frequency for any candidate cancer gene needs to be evaluated in each specific geographical area (Casula et al., 2007).

Exon	DNA change	p16INK4A change	p14ARF change	References
1α	c.68G>A	p.Gly23Asp	/	Fargnoli et al., 1998; Scaini et al., 2009
1α	c.67G>A	p.Gly23Ser	/	Gensini et al., 2007
1α	c.71G>C	p.Arg24Pro	/	Harland et al., 1997; Fargnoli et al., 1998
1α	c.79G>T	p.Glu27X	/	Ghiorzo et al., 2006
1α	c.142C>A	p.Pro48Thr	/	Della Torre et al., 2001
1β	g.161G>A	/	p.Arg54His	Binni et al., 2010
2	c.172C>T	p.Arg58X	p.Pro72Leu	Bruno et al., 2009
2	c.194T>C	p.Leu65Pro	p.= (Ala79)	Landi et al., 2004
2	c.202_203GC>TT	p.Ala68Leu	p.Arg82Leu	Bruno et al., 2009
2	c.212A>T	p.Asn71Ile	p.Gln85His	Fargnoli et al., 1998
2	c.229A > G	p.Thr77Ala	p.His91Arg	Pastorino et al., 2008
2	N.A.	p.Ala86Thr	N.A.	Bruno et al., 2009
2	c.281T>C	p.Leu94Pro	p.= (Ala108)	Bruno et al., 2009
2	c.301G>T	p.Gly101Trp	p.Arg115Leu	Ghiorzo et al., 1999
2	c.341C>T	p.Pro114Leu	p.= (Ala128)	Fargnoli et al., 1998
2	c.377T>A	p.Val126Asp	/	Bruno et al., 2009
2	c.379G>C	p.Ala127Pro	/	Bruno et al., 2009

Table 1. Pathogenic CDKN2A missense mutations in Italy (N.A.: *N*ot *A*vailable in literature).

5.2.1 North-western Italy

The purpose of a recent study (Pastorino et al., 2008) was the analysis of the contribution of CDKN2A mutations and MC1R variants to the development of *M*ultiple *P*rimary *M*elanoma (MPM) versus *S*ingle *P*rimary *M*elanoma (SPM) in the population of two different towns of North-western Italy. As for MC1R, which is the topic of the present paragraph, thirty different non-synonymous variants were found with an overall allele frequency of 56.32% in MPM patients and 44.85% in SPM patients. In both MPM and SPM patients, V60L and R151C were the most frequently detected variants. Five novel variants (R67W, L100P, D184G, I221T and 1339 + 5 C >T) and two other novel variants (A149T and V156A) were detected in the MPM and in the SPM cases, respectively. No association was observed between the presence of MC1R variants and age at diagnosis. Compared to the SPM patients, MPM cases had a 2-fold increased likelihood of being MC1R variant carriers and a higher probability of carrying two or more variants, as reported by Kanetsky et al. (2006) in their large multicenter population-based study.

Finally, the analysis showed no specific association between the variant type and the number of CMs (one, two or more). This finding suggested that the presence of at least one MC1R variant, regardless of whether it is a R or a r variant, may influence the probability of developing two or more CMs, independently of the CDKN2A mutation status. This consideration was supported also by the results on the association between phenotypic characteristics and prevalence and type of MC1R variants: unexpectedly, the r rather than the R variants were associated with light eyes and hair. Fair skin was significantly associated with both r and R variants. At the very end, the results on MC1R variants in SPM/MPM patients suggest that, in this population, the number rather than the type of MC1R variants increases the risk of developing MPM.

5.2.2 North-eastern Italy

A case–control study including 183 incident cases of any stage and 179 controls was conducted in North-eastern Italy to identify important risk factors and determine how their combinations affected risk in a Mediterranean population (Landi et al., 2001). Presence of dysplastic nevi, low propensity to tan, light eye, and light skin colour were significantly associated with melanoma risk after adjustment for age, gender and pigmentation characteristics, showing the need of preventive advice against melanoma in these populations. According to the combination of these factors, a relative risk range from 1 to 98.5 was found. Moreover, light skin colour, high number of sunburns with blistering, and low propensity to tan were significantly associated with melanoma thickness, possibly indicating that individuals with these characteristics underestimate their risk and seek attention when their lesion is already advanced.

5.2.3 Central Italy

A work by Fargnoli and coll. (2006) investigated the contribution of the MC1R genotype to the risk of sporadic cutaneous melanoma in a population of Central Italy composed by 100 patients with sporadic cutaneous melanoma of any stage and 100 unrelated controls. All high-penetrance R variants of MC1R combined conferred a 2.5-fold increased risk of melanoma, and a significant increase in melanoma risk associated with high-penetrance R variants was observed mainly in the presence of clinically atypical nevi, more than 50

melanocytic nevi and prolonged UV exposure habits. Consistent with other studies, the R151C allele was significantly associated with melanoma risk, conferring a 2.9-fold higher risk. Interestingly, D294H was detected only in melanoma patients, and not in controls, although a larger sample size would be required to achieve statistical significance. Finally, the data confirmed the role of high penetrance R variants in the genetic predisposition to sporadic melanoma in an Italian population.

5.2.4 Southern Italy

Oncogenic BRAF signalling was demonstrated to interfere with the CDKN2A activity. A sustained expression of the mutated BRAF protein induces p16 expression and cell cycle arrest, indicating that both BRAF and CDKN2A pathways are functionally associated (Michaloglou et al., 2005). Finally, inherited mutations of the BRCA2 gene give rise to a multi-site cancer phenotype which includes ocular and cutaneous melanomas in addition to the main predisposition to breast (in females and males) and ovarian cancers. The work of Casula and coll. (2007) tried to assess the likelihood of identifying CDKN2A mutations in patients, belonging to Southern Italy or Sardinia, with histologically-proven diagnosis of melanoma, included regardless of age at diagnosis, family history status, and disease features.

The authors made also a final comparison between prevalence of CDKN2A germline mutations within different Italian regions, pooling together data coming from different publications: in contrast to a higher frequency of CDKN2A germline mutations observed in non-familial cases from Northern Italy, their findings among the same type of patients from Southern Italy strongly suggest that the discrepancy in CDKN2A mutation frequency may be due to patient origin and/or to the different 'genetic background' of the population.

Molecular analysis was also performed in order to identify any correlation between genetic alterations and phenotypic parameters: CDKN2A mutations were more frequent in patients with familial history of melanoma compared to patients without. Moreover, age at diagnosis was significantly correlated with the presence of a CDKN2A mutation: the mean age of onset was significantly lower in carriers of mutations compared to non-carriers.

To evaluate whether additional candidate genes might be involved in melanoma susceptibility, prevalence of germline mutations in BRAF and BRCA2 genes (the other two major genes related to melanoma pathogenesis) was assessed in subsets of patients originating from different geographical areas within Southern Italy.

Germline mutations in BRAF and BRCA2 genes were found in Sardinian patients only (altogether, 3/116; 2.6%), with no additional alterations in the remaining cases from Southern Italy. These findings strongly confirmed that mutation frequency for any candidate cancer gene needs to be evaluated in each geographical area.

6. Conclusions

Familial melanoma has proven to be a disease with a heterogeneous etiology, associated with mutations in susceptibility genes. Several high and moderate/low risk genes have now been identified. CDKN2A remains the most important high risk gene in melanoma development; other predisposition genes comprise CDK4, MC1R, and a variety of risk modifier genes including MTAP, EGF, GST, MDM2 and many others. There is a great

interest in the identification of high risk individuals harbouring melanoma-associated gene mutations to offer clinical screening and follow up, since early detection and diagnosis are the most significant tools in improving melanoma prognosis and outcome. Undoubtedly, the identification of new predisposition loci, together with further investigations on gene-gene and gene-environment interactions will shed light on molecular mechanisms involved in melanocyte transformation. Of course, the real challenge of melanoma research relies in converting the growing body of information on the disease into effective strategies to implement melanoma prevention and treatment in the near future.

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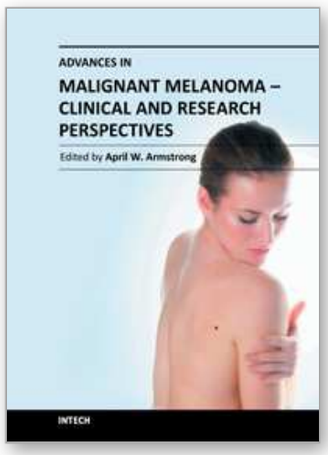

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This book titled Advances in Malignant Melanoma - Clinical and Research Perspectives represents an international effort to highlight advances in our understanding of malignant melanoma from both clinical and research perspectives. The authors for this book consist of an international group of recognized leaders in melanoma research and patient care, and they share their unique perspectives regarding melanoma epidemiology, risk factors, diagnostic and prognostic tools, phenotypes, treatment, and future research directions. The book is divide into four sections: (1) Epidemiology and Risk Factors of Melanoma, (2) Clinical Phenotypes of Melanoma, (3) Investigational Treatments for Melanoma and Pigmentary Disorders, and (4) Advances in Melanoma Translational Research. This book does not attempt to exhaustively cover all aspects of the aforementioned topics. Rather, it is a compilation of our authorsâ€™ pearls and unique perspectives on the relevant advances in melanoma during the recent years.

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