University of Siena

Ph.D in Medical Genetics

Comprehensive analysis of the CDKN2A gene mutations in Familial and Multiple Primary Cutaneous Melanoma.

Dr. Marina Vignoli

Supervisor
Prof. Maurizio Genuardi

Coordinator
Prof. Alessandra Renieri

Academic Year 2008-2009

INDEX

INTRODUCTION

Familial and Multiple Primary Cutaneous Melanoma

Melanoma is a malignant tumor that originates from pigment-producing melanocytes. Melanocytes arise from neural crest–derived progenitor cells that migrate into the skin from the central nervous system. These cells are distributed at the junction of the epidermal and dermal layers of the skin. The amount of melanin produced by melanocytes dictates the degree of skin pigmentation. Melanomas can arise from noncutaneous melanocytes found in the lining of the choroidal layer of the eye, the meninges, and the gastrointestinal and genitourinary tracts, but the majority of these tumors occur as lesions anywhere on the skin. The lungs, liver, and brain are the preferred sites for metastases.

Cutaneous malignant melanoma (CMM) is the most prevalent form in sun-rich regions populated by light-skinned individuals, in particular Australia, southern Europe, South Africa, and the southern United States. Its incidence has been rising steadily over the past 20 years in white-skinned populations and its etiology is heterogeneous and complex. In Italy, between 2003 and 2005, an annual average of 14.3 cases per 100,000 male-years and 13.6 cases per 100,000 female-years were registered (http://www.registri-tumori.it/PDF/AIRTUM2009Trend/ E&P33_4-5S1_54_melanoma.pdf).

Sun exposure is the major environmental risk factor, while other relevant risk factors are host-related and include ethnicity, pigmentary characteristics, skin reactions to sunlight, increased numbers of banal or atypical nevi, and family history (Armstrong and English, 1996; Chaudru et al., 2005).

The cumulative risk of developing CMM in the Italian population ranges between 0.2% and 0.8% (Balzi et al., 1997). The majority of melanoma cases are sporadic, while approximately 10% occur in a familial setting (Greene, 1999). Individuals with a significant family history of melanoma have a 30- to 70-fold increased risk of developing the disease compared to the general population (Kefford et al., 1999).

The definition "familial cutaneous malignant melanoma" (FCMM) is used to indicate families in which either two or more first-degree relatives are diagnosed with melanoma, or families with three or more melanoma patients irrespectively of the degree of relationship.

Patients with a positive family history are at significantly greater risk of developing multiple primary melanomas (MPM) (Ferrone et al., 2005). Moreover, with the increasing number of patients surviving this tumor, the fraction of individuals who develop more than one melanoma is rising. Indeed it has been estimated that 3% to 5% of all patients with CMM will develop additional primary CMMs in their lifetime (Leachman et al., 2009).

Individual and familial clustering of melanomas can be explained by both non-genetic and genetic factors, but many cases of concurrent MPM and FCMM are likely to be caused by hereditary susceptibility to the disease.

The classic family history features that raise the possibility of an inherited cancer syndrome are multiple affected family members (particularly with a

vertical pattern of inheritance), occurrence of cancer types known to be associated with a specific hereditary syndrome, presence of individuals given the diagnosis of multiple primary cancers, and early age of onset.

Analysis of large well-defined pedigrees has allowed to estabilish that trasmission of susceptibility to melanoma is inherited as an autosomal dominant trait with incomplete penetrance and variable expressivity.

Genetic linkage and candidate gene analyses in melanoma families worldwide have identified cyclin-dependent kinase inhibitor 2A (*CDKN2A*) as the major high penetrance susceptibiliy gene and cyclin-dependent kinase 4 (*CDK4*) as the second known high-risk melanoma susceptibility gene.

Germline mutations in *CDKN2A* are reported to be present in 20% to 40% of families with three or more CMM cases (Goldstein and Tucker, 1997).

To date, worldwide studies suggest that the presence and frequency of *CDKN2A* mutations in familial melanoma vary between countries and across groups in the same continent, depending on factors such as baseline melanoma incidence rates and family and population selection in studies. A collaborative study by 17 research groups belonging to the International Melanoma Genetics Consortium (GenoMEL) showed that overall 39% of the families were mutation-positive, ranging from 20% in Australia, to 45% in North America and 57% in Europe (Goldstein et al., 2007).

Additionally, the number of affected members, age at first diagnosis, the presence of multiple primary melanomas and the presence of associated cancers in the family show significant associations with *CDKN2A* mutations, but the effects vary widely across continents (Bruno et al., 2009).

The number of melanoma patients per family is the first feature that influences the chance of detecting a *CDKN2A* mutation. In some European studies mutations are found in kindreds with only two melanoma cases, as well as in kindreds with larger numbers of melanoma cases (Soufir et al., 1998; Fargnoli et al., 1998; Ghiorzo et al., 1999), while in other geographic areas *CDKN2A* mutations have been mostly recorded in larger families (Gruis et al., 1995a; Borg et al., 1996; Platz et al., 1997; Bishop et al., 1999; Ruiz et al., 1999). However all studies confirmed that the likelihood of *CDKN2A* mutation detection increases with the number of melanomas in the family.

As with family history, the prevalence of *CDKN2A* mutations raises with increases in the numbers of both MPMs per patient and of family members with MPMs. Studies of patients having ≥4 melanomas indicate a 29% to 100% likelihood of finding a mutation, at least in low incidence countries (Leachman et al., 2009; Bruno et al., 2009).

In general, familial melanoma cases appear to have an earlier age at diagnosis than nonfamilial cases, and the incidence of *CDKN2A* mutations is higher in families with an early age at onset (Della Torre et al., 2001; Holland et al., 1999).

In addition to melanoma, the presence of pancreatic cancer is strongly associated with *CDKN2A* mutation in a subset of melanoma-prone families ($P<0.0001$). In these cases, the likelihood of finding a mutation ranges from 38% to 72%. (Goldstein et al., 2006).

Although *CDKN2A* mutations confer substantial risk for melanoma, not all carriers who are heterozygotes develop CMM. By age 80 years, an individual ascertained from multiple-case families with a *CDKN2A* mutation has an

increased risk of developing melanoma of 58% in Europe, 76% in the United States and 91% in Australia (Bishop et al., 2002). As observed in other inherited cancer conditions such as breast cancer (Antoniou et al., 2003), *CDKN2A* mutation carriers in the overall population have a much lower melanoma risk than carriers within multiple case families (Begg et al., 2005). However, gene penetrance estimated on population-ascertained mutation carriers, although considerably lower, is still substantial (28% by age 80 years) (Begg et al., 2005).

Overall, the risk appears to vary between countries and families, and it is not yet clear whether this variation results from the type of mutation, environmental exposures, coinheritance with other genetic variations, or other not yet identified genetic variables. Previous family-based studies (Goldstein et al., 2005; Chaudru et al., 2005) have identified atypical/dysplastic nevi, high numbers of typical nevi, poor tanning ability, or sunburns as clinical/environmental risk factors in the development of melanoma in families with *CDKN2A* mutations.

It has been observed that CMM risk associated with *CDKN2A* mutations can be enhanced also by other known genetic factors acting as modifiers, such as *MC1R* variants.

The *MC1R* gene (16q24), which encodes for the melanocyte-stimulating hormone receptor 1 and is implicated in the pigmentation process, has been classified as a low-risk melanoma susceptibility gene (Hayward et al., 2003) and shown to be a risk factor for melanoma in families segregating *CDKN2A* mutations (Goldstein et al., 2005; Chaudru et al., 2005; Box et al., 2001; Van der Velden et al., 2001). Its role in melanoma development was assessed by

Goldstein et al. (2007), who evaluated the distribution of *MC1R* variants in patients from France, Italy, Spain, and the United States who were heterozygotes for the same *CDKN2A* mutation (p.G101W). This study showed a significant association between increased number of *MC1R* variants and melanoma risk, although Italian melanoma cases had fewer *MC1R* variants than cases from the other nations. However, previous studies of unselected melanoma cases from different Italian regions have detected significant associations between *MC1R* variants and melanoma risk (Pastorino et al., 2004; Landi et al., 2005; Fargnoli et al., 2006a; Fargnoli et al., 2006b). Finally, there was also a significant decrease in median age at melanoma diagnosis as the number of *MC1R* variants increased. Age at diagnosis was also related to the presence of specific *MC1R* variants (Goldstein et al., 2007).

A very recent Italian collaborative study that comprised 9 centers was conducted on 208 Italian melanoma families that underwent clinical genetic counseling and testing.

Overall 33% of the families carried mutations in *CDKN2A*, and no significant difference in the mutation rate was found between families with one and with two tested relatives affected with CMM, supporting the hypothesis that in a country of low CMM incidence such as Italy, the likelihood of chance clustering of cases in a family is relatively low (Bruno et al., 2009).

The *CDKN2A* mutation frequency in Italian families with two affected members was 25%, thus a substantial subset of *CDKN2A* mutation-positive families would not be identified if more conservative criteria for access to testing were adopted in Italy, such as the presence of at least 3 affected members. This result underscores the importance of including the geographic

location in the assessment of candidacy to genetic counseling. The mutation rate observed in families with 3 or more CMM cases was 54%, similar to the rate seen among the European families in the GenoMEL study, which has been designed using more stringent selection criteria (presence of at least 3 affected members).

Patients from *CDKN2A* mutation-positive families had a significantly younger median age at first melanoma diagnosis compared with patients who belonged to families with no mutations (42 vs 49 years, $P < 0.0001$) (Bruno et al., 2009).

In addition 23% of mutation positive individuals had developed MPM versus 9% of those who did not carry *CDKN2A* mutations ($P=0.0002$). The frequency of mutations increased significantly with the number of patients with MPM in the family and reached 100% in the families with two or more MPMs, (Bruno et al., 2009) confirming that the number of cases with MPM increases the likelihood of detecting a germline *CDKN2A* mutation in a family (Goldstein et al., 2006; Monzon et al., 1998; Mantelli et al., 2002; Puig et al., 2005).

Previously, in another Italian study, it had been found that MPM cases had a 4-fold higher likelihood of carrying a *CDKN2A* mutation than single primary melanoma cases, regardless of family history (Pastorino et al., 2008).

These findings provide support to the selection criteria for *CDKN2A/CDK4* clinical genetic testing recommended by the Italian Society of Human Genetics (SIGU), which include either 1) two or more CMM cases among first-degree relatives, or 2) three or more CMM-affected family members (in the same branch of the family).

Cyclin-dependent kinase inhibitor 2A: CDKN2A

The human *CDKN2A*, that was designated p16(INK4), was cloned in 1993 (Serrano et al., 1993) using cyclin-dependent kinase 4 in a yeast 2-hybrid screen; the inferred 148-amino acid protein had an estimated molecular mass of 15.8 kD.

In 1994 Kamb et al. identified a putative tumor suppressor locus on chromosome band 9p21, within a region of less than 40 kb, following a search for homozygous deletions in melanoma cell lines. The region was found to contain a gene, called *MTS1* (for multiple tumor suppressor-1), that encoded a previously identified inhibitor of CDK4 (p16). The sequence of the *MTS1* gene as determined by Kamb et al. (1994) was identical to that of the *CDKN2A* gene previously identified by Serrano et al. (1993).

CDKN2A is located on the short arm of chromosome 9 (9p21) and gives rise to 2 distinct transcripts from different promoters. Each transcript has a specific 5' exon, designated exon 1α and exon 1β; the latter is located approximately 20 kb centromeric to exon 1α. The alpha transcript comprises exons 1α, 2, 3, and encodes a low–molecular weight protein of 156 aminoacids, p16INK4A, while the smaller beta transcript comprises exons 1β, 2 and 3, and encodes the alternative protein product p14ARF of 173 aminoacids. The two different proteins are translated in alternate reading frames.

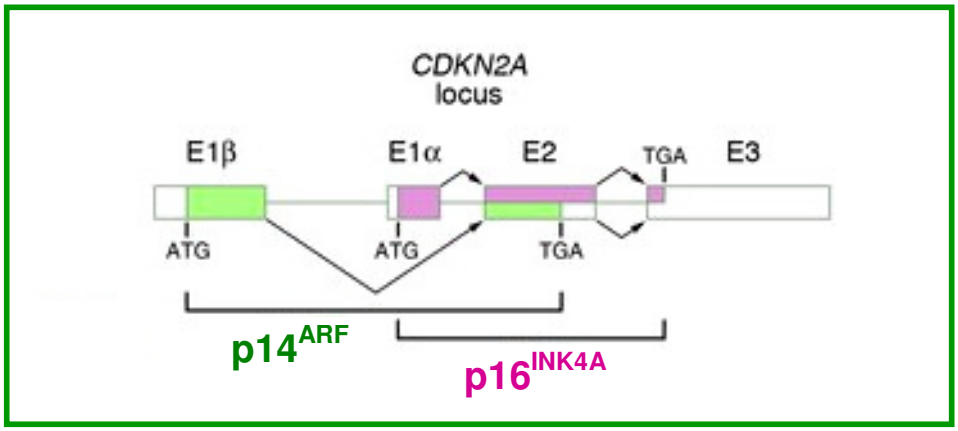

Fig.1 Schematic structure of *CDKN2A* locus encoding p14ARF and p16INK4A from alternatively spliced exons.

The frequent deletion or mutation of *CDKN2A* in tumor cells suggests that p16INK4A acts as a tumor suppressor. Lukas et al. (1995) showed that wild type p16INK4A arrests normal diploid cells in late G1, whereas a tumor-associated mutant of p16INK4A does not. Significantly, the ability of p16INK4A to induce cell cycle arrest was lost in cells lacking functional retinoblastoma protein (RB1). Thus, loss of p16INK4A, overexpression of D-cyclins, and loss of retinoblastoma have similar effects on G1 progression, and may represent a common pathway to tumorigenesis. The mutation tested by Lukas et al. (1995) in their studies (p.P114L) had been observed in 3 independent melanoma cell lines. In 1995 Koh et al. demonstrated that p16INK4A can act as a potent and specific inhibitor of progression through the G1 phase of the cell cycle and that several tumor-derived alleles of p16INK4A encode functionally compromised proteins. *In vivo*, the presence of functional retinoblastoma protein appeared to be necessary but may not be sufficient to confer full sensitivity to p16INK4A-mediated growth arrest.

The p16INK4A protein belongs to the INK4 family of CDK inhibitors that interact directly with the CDKs, CDK4 and CDK6, and block their association with D-type cyclins, thereby preventing the formation of CDK/Cyclin D complexes (Serrano et al., 1993). These complexes phosphorylate the retinoblastoma protein, allowing the cell to progress through the G1 cell cycle checkpoint (Serrano et al., 1995). Thus, p16INK4A acts as a tumor suppressor and negatively regulates cell growth by arresting cells in the G1 phase.

In physiological settings, increased expression of p16INK4a is associated with growth arrest that can be permanent such as in replicative senescence, or transient as a response to constitutive Ras-Raf-MEK signaling in somatic cells. Thus, loss of p16INK4a activity would enable incipient tumor cells to evade senescence and in general cell cylce arrest.

Ohtani et al. (2001) demonstrated a role for the ETS1 and ETS2 transcription factors in regulating the expression of *CDKN2A,* in the above mentioned different contexts, based on their ability to activate its promoter through an ETS binding site and on their patterns of expression during the life span of human diploid fibroblasts. The induction of *CDKN2A* by ETS2, which is abundant in young human diploid fibroblasts, is potentiated by signaling through the Ras-Raf-MEK kinase cascade and inhibited by a direct interaction with the helix-loop-helix protein ID1. In senescent cells, where the ETS2 levels and MEK signaling decline, the marked increase in p16(INK4A) expression is consistent with the reduction of ID1 and accumulation of ETS1.

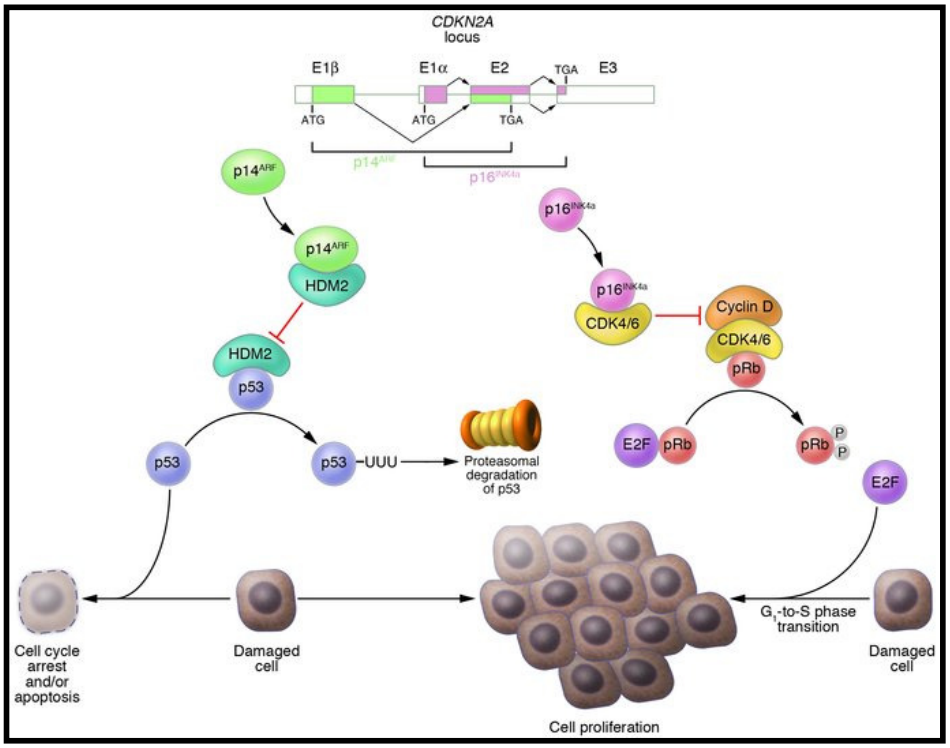

Fig.2 Schematic representation of p16INK4A and p14ARF role in cell cycle regulation.

The four INK4 proteins, p16INK4A, p15INK4B, p18INK4C, and p19INK4D, are biochemically and structurally related, and orthologs have been identified in a variety of species. Three dimensional (3D) structures of p16INK4A bound to CDK4 (Byeon et al., 1998) and p16INK4A bound to CDK6 (Russo et al., 1998) have been determined by X-ray crystallography and nuclear magnetic resonance (NMR). The 156 residues of human p16INK4A essentially comprise four ankyrin repeats (I–IV), a structural motif of about 33 amino acids found in many diverse proteins. Typically, adjacent repeats interact to form helix-loop-helix modules linked by β-hairpins (Zhang and Peng, 2000) and in the case of

p16INK4A it appears that all four repeats are needed to sustain the interaction with CDK4 and CDK6 (Yang et al., 1995; Lilischkis et al., 1996).

On the other hand, p14ARF acts via the p53 pathway to induce cell cycle arrest or apoptosis (Zhang et al., 1998; Pomerantz et al., 1998). Zhang et al. (1998) proposed that both unrelated proteins encoded by the *CDKN2A* locus function in tumor suppression. They showed that p14ARF binds to MDM2 and promotes its rapid degradation. This interaction is mediated by the exon 1β-encoded N-terminal domain of ARF and a C-terminal region of MDM2. ARF-promoted MDM2 degradation is associated with MDM2 modification and concurrent p53 stabilization and accumulation. The functional consequence of ARF-regulated p53 levels via MDM2 proteolysis is evidenced by the ability of ectopically expressed ARF to restore p53-imposed G1 cell cycle arrest, that is otherwise abrogated by MDM2. Thus, Zhang et al. (1998) concluded that deletion of the *CDKN2A* locus simultaneously impairs the INK4A--cyclin D/CDK4--RB and the ARF--MDM2--p53 pathways.

Zhang and Xiong (1999) reported that the human p14ARF protein predominantly localizes to the nucleolus via a sequence located within the exon 2-encoded C-terminal domain and is induced to leave the nucleolus by MDM2. p14ARF forms nuclear bodies with MDM2 and p53 and blocks p53 and MDM2 nuclear export. Tumor-associated mutations in exon 2 of the *CDKN2A* locus disrupted the nucleolar localization of p14ARF and reduced its ability to block p53 nuclear export and to stabilize p53. These results suggested a

p14ARF-regulated MDM2-dependent p53 stabilization and linked the human tumor-associated mutations in the *CDKN2A* locus with a functional alteration.

Both p14ARF and p16INK4a proteins are nearly ubiquitously expressed. p14ARF mRNA is detectable in all tissues except pancreas and skeletal muscle, while p16INK4a mRNA is detectable in all tissues except brain and skeletal muscle.

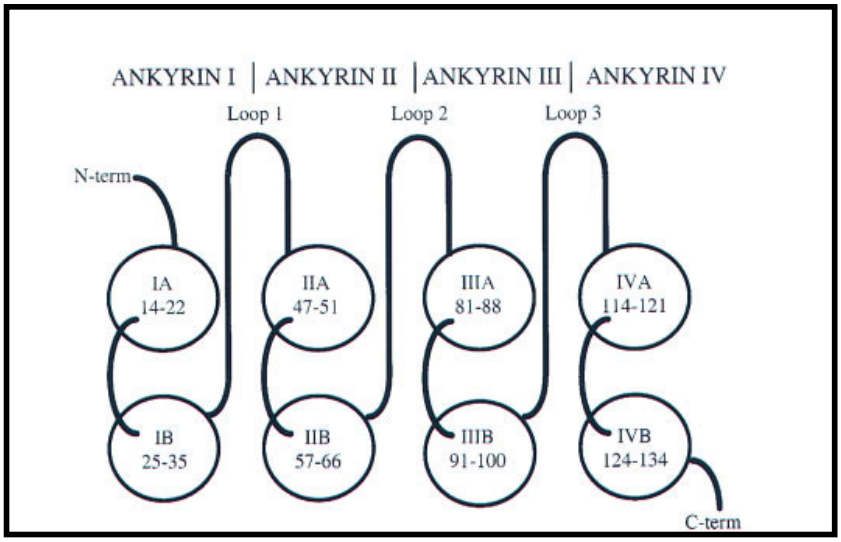

Fig.3 Topology diagram of the p16 structure with ankyrin repeats. Helices (in circle) are perpendicular to the plane of the page, and the residue numbers forming the helices are indicated.

CDKN2A mutation frequency and spectrum

Most *CDKN2A* germline mutations identified to date are located within the coding sequence, and the majority are single base pair substitutions in exons 1α and 2 mainly affecting p16INK4A function. Mutations in non coding regions, such as deep intronic regions and the promoter/5' UTR (Liu et al., 1999; Harland et al., 2001; Majore et al., 2004; Harland et al., 2005a), have also been described, but they only account for a small subset of all 9p21-linked families. To date, p14ARF-only mutations are limited to a few melanoma families and consist of splice site and insertions/deletions mutations (Hewitt et al., 2002; Randerson-Moor et al., 2001; Rizos et al., 2001b; Laud et al., 2006).

The most extensive study on the role of *CDKN2A* in familial melanoma was conducted by the Melanoma Genetics Consortium (GenoMEL; http://www.genomel.org), which comprises 17 centers from Europe, Australia, North America and the Middle East (Goldstein et al., 2006). The study included 466 melanoma-prone families with 2,137 CMM patients. Overall, 38% of families (n = 178) had *CDKN2A* mutations that involved the p16INK4A protein. Sixty-five percent of these mutations were missense mutations, 23% were insertions or deletions, 5% each, nonsense or splicing mutations, and 2% were regulatory mutations. In contrast, the seven p14ARF mutations (1,5%) were either novel splicing mutations (Harland et al., 2005b) or large deletions (Randerson-Moor et al., 2001; Laud et al., 2006). They also reported striking differences in the frequencies and distributions of mutations across geographic areas (Goldstein et al., 2006).

A significant proportion of *CDKN2A* mutations occur in the shared exon 2, potentially affecting the coding sequence of both products.

A few exon 2 germline mutations have been reported to impair both p14ARF and p16INK4A function. (Rizos et al., 2001a; Hashemi et al., 2002). In the GenoMEL study (Goldstein et al., 2006), 8 out of 22 missense mutations in exon 2 had no predicted effect on p14ARF, and no mutations affected p14ARF without affcting p16INK4a.

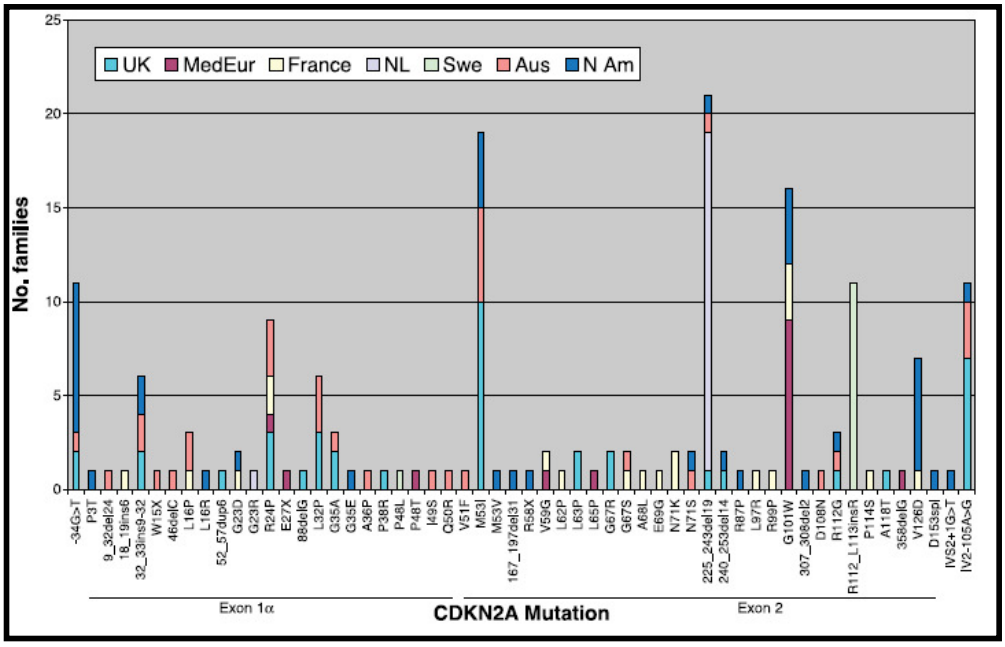

Fig.1 Number of families with *CDKN2A* mutations, differences according to geographic locales. Mutation are more frequently in exon 1α and 2.

- ***Founder Mutations***

The founder effect is a special case of genetic drift and occurs when a new population is established by a very small number of individuals from a larger population.

When a newly formed colony is small, its founders can strongly affect the population's genetic make-up far into the future. In humans, which have a slow reproduction rate, the population will remain small for many generations, effectively amplifying the drift effect generation after generation until the population reaches a certain size. Alleles which were present but relatively rare in the original population can move to one of two extremes. The most common one is that the allele is soon lost altogether, but the other possibility is that the allele survives and within a few generations has become much more diffuse throughout the population.

Founder mutations originate in long stretches of DNA on a single chromosome and, indeed, the original haplotype is the whole chromosome. As the generations progress, the proportion of the haplotype that is common to all carriers of the mutation is shortened (due to genetic recombination). Thus, the analysis of haplotypes allow to trace the founder mutation origin and the age of the founder mutation can be estimated by the length of the common haplotype: the longest is the haplotype, the youngest is the origin of the mutation.

Recurrent mutations of *CDKN2A* have been reported in different families worldwide, and haplotype analysis has been applied to determine whether these have arisen because of a common founder or because of multiple independent mutational events (Pollock et al., 1998; Majore et al., 2004). A total of 11

different founder mutations of either *CDKN2A*/p16INK4A or *ARF*/p14ARF have been described among the 125 *CDKN2A* germline mutations (missense, nonsense, frameshift, duplication or insertion, regulatory, splice, deletion) reported to date in the UVM database (https://biodesktop.uvm.edu/perl/p16) (Kannengiesser et al., 2007). Most of these mutations observed worldwide can be traced back to European countries (Goldstein et al., 2006).

Single founder *CDKN2A* mutations were the predominant mutations in Sweden, p.R112_L113insR or c.331_332insGTC (Hashemi et al., 2001), comprising 92% of observed families with mutations, and the Netherlands, c.225_243del19 (Gruis et al., 1995b), involving 90% of reported families with mutations. France and Mediterranean Europe (Spain/Italy) had the same most frequent *CDKN2A* founder mutation, p.G101W (Ciotti et al., 2000), encompassing 15% and 60% of families with mutations, respectively. Similarly, Australia and United Kingdom had the same most common recurrent *CDKN2A* mutations, p.M53I, c.IVS2-105a>G, p.R24P, and p.L32P (Harland et al., 2000), comprising 42% and 50% of observed families with mutations, respectively, which was not unexpected as the ancestry of long-term residents of Australia is predominantly British.

Founder or recurrent mutations seem to underlie susceptibility in the majority of familial melanoma cases in Italy, and may explain in part the high frequency of *CDKN2A* mutations in Italian two-case families (Bruno et al., 2009).

- ***Large rearrangements***

The question of whether or not large genomic deletions, undetectable by traditional PCR-amplification and sequencing of individual exons, may be the cause of some familial cases where no *CDKN2A* mutations are found, has been raised, and few authors have searched for large *CDKN2A* deletions or rearrangements (Fitzgerald et al., 1996; Bahuau et al., 1998; Randerson-Moor et al., 2001; Debniak et al., 2004; Mistry et al., 2005; Knappskog et al., 2006; Erlandson et al., 2007). To date, germline large deletions have been identified in the 9p21 region in only six families worldwide. A deletion involving *CDKN2A* exon1α, 2, and 3 and a deletion removing exon 1α and half of exon 2 were described in two melanoma-prone kindreds, originated from UK and from Norway, respectively (Mistry et al., 2005; Knappskog et al., 2006). Large deletions have also been found in families with combined proneness to melanoma and nervous system tumours (NST): a gross deletion ablating the whole *CDKN2A* and *CDKN2B* genes has been reported in a French family (Bahuau et al., 1998; Pasmant et al., 2007), and a deletion of p14ARF-specific exon 1β of the *CDKN2A* gene has been found in one US family and in two UK families (Bahuau et al., 1998; Randerson-Moor et al., 2001; Mistry et al., 2005; Laud et al., 2006). A large duplication of the *CDKN2A/CDKN2B* loci has also been reported in a melanoma patient from a Swedish family, but the clinical significance of this variant is not evident (Erlandson et al, 2007).

Cyclin-dependent kinase 4: CDK4

Cyclin-dependent kinase-4 (CDK4) is a protein-serine kinase involved in the cell cycle. The sequential activation of cyclin-dependent kinases and their subsequent phosphorylation of critical substrates promote orderly progression through the cell cycle.

The complexes formed by CDK4 and the D-type cyclins are involved in the control of cell proliferation during the G1 phase. CDK4 is inhibited by p16INK4A.

The gene encoding cyclin-dependent kinase-4 *CDK4* is located on chromosome 12q13; it spans 5 kb and contains 8 exons.

Mutation frequency and spectrum

In contrast to *CDKN2A*, only a few (less than 15) melanoma families harboring *CDK4* mutations have been reported worldwide to date (Helsing et al., 2008; Molven et al., 2005; Pjanova et al., 2009; Soufir et al., 1998; Zuo et al., 1996).

To date, the only two *CDK4* germline mutations found in melanoma patients (Arg24His and Arg24Cys) have been identified in exon 2, which codes for the p16 binding site (Zuo et al., 1996; Soufir et al., 998; Molven et al., 2005). These mutations are also the most common somatic *CDK4* mutations found in melanoma (Bennett, 2008; Dahl and Guldberg, 2007; de Snoo and Hayward 2005; Forbes et al., 2008).

In a Norwegian population-based study, three out of 390 patients diagnosed with MPM carried a *CDK4* mutation (0.8%), and these patients were all later confirmed to belong to a large melanoma-prone family (Molven et al., 2005).

MATERIALS AND METHODS

Patient and Samples

The Medical Genetics Unit of the University of Florence routinely provides genetic counseling and testing to multiple primary melanoma patients (with ≥2 primary melanomas) and probands with ≥2 relatives affected with CMM in the same branch. Familial or MPM patients from medical genetics units of the Catholic University, Policlinico A. Gemelli, in Rome and from the University of Siena, Policlinico Le Scotte, are referred to Florence for genetic testing.

The study included 66 families referred to the Medical Genetics Unit of Florence, seven families referred from Rome and one family referred from Siena in the period 2004-2009. Overall, 74 unrelated probands were enrolled in this study. Written informed consent to genetic investigations, approved by the local ethics committees, was obtained from each patient. Fourty-nine patients come from melanoma-prone families, 18 of these were also affected by MPMs; the remaining 25 probands were sporadic MPM cases without affected relatives (Fig.1). Moreover, in 6 FCMM patients melanoma was associated with pancreatic cancer.

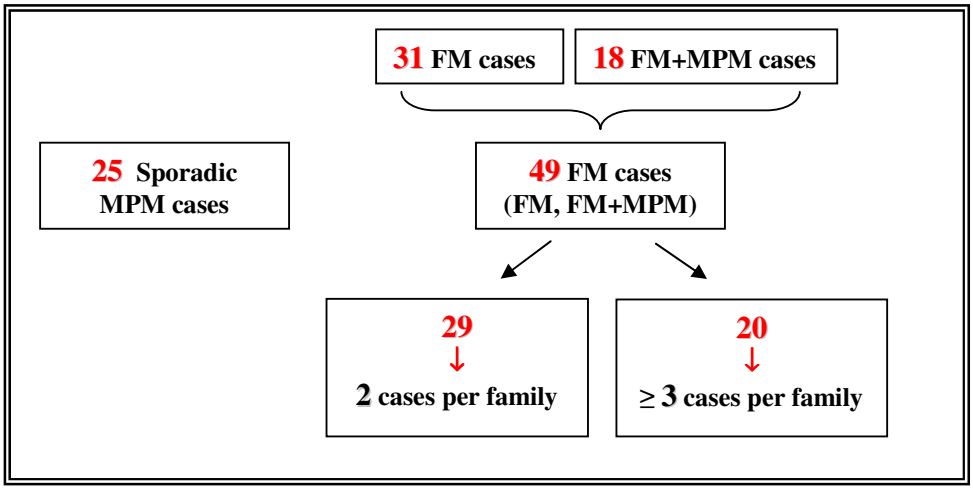

Fig.1 Number of patients and subgroups assigning. FM: familial melanoma cases; FM+MPM: MPM case with a positive family history. Number of families with 2 or with ≥ 3 affected members is reported.

Twenty out of 32 familial melanoma cases from Florence have also been included in a screening for large *CDKN2A* rearrangements that was conducted among melanoma families without detectable *CDKN2A* and *CDK4* point mutations. This study included 118 families referred to five medical genetics or cancer genetics centres in Florence, Genoa, Milan (European Institute of Oncology, IEO, and Fondazione IRCCS Istituto Nazionale dei Tumori, INT), and Padua until July 2007. Six additional families included in the study were from IMI (Italian Melanoma Intergroup) centers (Aviano, Turin, Pisa, and Naples) and had been referred to Genoa for counseling and testing. Overall, 124 unrelated probands were enrolled in this study. Families were considered to be eligible for this part of the study when ≥2 CMM cases were present.

Blood samples from 141 individuals without a family history of melanoma, originary from Tuscany, were used to estimate frequencies of the p.G23S mutation and of microsatellite marker alleles in the general population, as well as to determine control haplotypes. Informed consent was obtained from all study participants.

DNA derived from the Jurkat cell line (New England Biolabs, Ipswich, MA) was chosen as a reference sample for Real Time gene dosage analysis since this cell line carries a homozygous deletion of the *CDKN2A* locus (Ogawa et al., 1994). In order to artificially reproduce a hemideleted sample, DNAs derived from the Jurkat cell line and from one of the above-mentioned control samples were mixed in 1:1 proportions.

Mutation Analysis of CDKN2A

Genomic DNA was extracted from peripheral blood leukocytes using a standard phenol/chloroform procedure and resuspended in 300-400 µl of TE buffer (pH 8).

CDKN2A exons 1β, 1α, 2, 3 and exon–intron borders were amplified by PCR and analysed by direct sequencing using primers reported in Tab.1. PCR was carried out in a final volume of 50 µl containing 100 ng of genomic DNA template, 5 µl of PCR buffer 10X, 1.5 mM $MgCl_2$, 5 µl of dNTPs (0.25mM each), 10 µM forward and reverse primer, and 2 U of Taq DNA polymerase.

For amplification, each sample was denatured at 95°C for 10 min and subjected to 30 amplification cycles of denaturation at 95°C for 30 s, annealing for 30 s and extension at 72°C for 30 s; final extensions were 72°C for 7 min.

PCR products were purified with Qiagen PCR purification kit (Qiagen, Milan, Italy). To perform the cycle sequencing reaction, 2 µl of purified DNA fragment was blended with each primer (0.8 mmol/l) in a Terminator Ready Reaction Mix containing Big Dye Terminators (Applied Biosystems, Foster City, California, USA), denatured for 5 min at 95°C and submitted to 30 cycles at 95°C for 30 s, 50°C for 10 s, and 60°C for 4 min. A second purification with DyeEx 2.0 Spin Kit (Qiagen) was performed for Big Dye removal. Five µl of marked and purified DNA were submitted to sequencing analysis on an ABI PRISM 310 Genetic Analyser (Applied Biosystems). Sequence variants were confirmed by both forward and reverse directions with primers used in the initial PCR amplification of each exon.

Exon	Primer	Sequence	Annealing temperature (°C)	PCR product (bp)
Ex 1β	P14 1BF P14 1BR	TCCCAGTCTGCAGTTAAGG GTCTAAGTCGTTGTAACCCG	60°C	446 bp
Ex 1α	P16 1AF P16 1AR	GAAGAAAGAGGAGGGGCTG GCGCTACCTGATTCCAATTC	58°C	340 bp
Ex 2	P16 2F P16 2R	AGCTTCCTTTCCGTCATG GGAAGCTCTCAGGGTACAAATTC	62°C	410 bp
Ex 3	P16 3F P16 3R	CCATTGCGAGAACTTTATCC TGGACATTTACGGTAGTGGG	56°C	328 bp
Ex 1β real time	1BetaF 1BetaR	GTTAAGGGGGCAGGAGTG GGGATGTGAACCACGAAA	56°C	171 bp
Ex 1α real time	1AlfaF 1AlfaR	GGTCGGGTAGAGGAGGTG CCAATTCCCCTGCAAACT	56°C	157 bp
Ex 2 real time	2F real 2R real	CTTCCTGGACACGCTGGT CATGGTTACTGCCTCTGGTG	60°C	161 bp
Ex 3 real time	3F real 3R real	AAGTATTTCAATGCCGGTAGG GGACCTTCGGTGACTGATGA	56°C	177 bp

Table 1. Primers and conditions used for PCR and quantitative Real-Time PCR.

Mutation nomenclature follows the Human Genome Variation Society (URL: http://www.hgvs.org/mutnomen/) recommendations.

Direct sequencing of *CDK4* exon 2 had been previously performed in Genoa on the 124 mutation-negative patients that were part of the multi-centers screening for large rearrangements in *CDKN2A* locus.

The presence of the p.G23S (c.67G>A) mutation was investigated by restriction-fragment length polymorphism- PCR in a series of 100 anonymized control subjects without family history of CMM among first-degree relatives. DNA samples were amplified using primers 50-GGGAGCAGCATGGAGCCG-30 (forward) and 50-AGTCGCCCGCCATCCCCT-30 (reverse). PCR products were subsequently digested with the restriction enzyme *AvaI* and visualized on a UV transilluminator following agarose gel electrophoresis and ethidum bromide staining. Wild-type alleles are cut into 132 and 71 bp fragments, whereas the c.67G>A allele is left undigested at the size of 203 bp.

Multiplex ligation-dependent probe amplification analysis

Multiplex ligation-dependent probe amplification analysis was performed using the 9p21 MLPA kit (P024B) (MRC-Holland, Amsterdam, Netherlands) according to the manufacturer's instructions. This probemix contains nine different probes for *CDKN2A* (five located in the four coding exons and four located in the promoter regions of either $CDKN2A^{ARF}$ or $CDKN2A^{INK4A}$), 15 probes for *CDKN2B*, *MTAP* and other surrounding genes in the 9p21 region, and 15 control probes located on different chromosomes. MLPA fragments were visualized on an ABI310 Automated Capillary DNA Sequencer (Applied Biosystems, Foster City, CA), using ABI POP-4 polymer, and GeneScan-

TAMRA 500 size standard (Applied Biosystems). Data were analysed with the ABI PRISM GeneScan software (Applied Biosystems) and gene dosage quotients were calculated using Coffalyser MLPA DAT Software (MRC Holland, www.mrc-holland.com/pages/indexpag.html). This software uses normalization against 15 autosomal control probes, considering peak areas as the quantitative measure of DNA content. For each *CDKN2A* fragment analyzed, samples were considered as wild-type (with two alleles), hemideleted (with one allele) or with a biallelic deletion when their quotients were 0.70–1.30, close to 0.5, and 0, respectively, as suggested by the manufacturer (www.mrc-holland.com/pages/coffalyser_pagepag.html).

Real time PCR analysis

Real Time quantitative PCR was performed for *CDKN2A* exons 1β, 1α, 2, 3 on a Rotor-Gene 6000 Instrument (Corbett Research, Sidney, Australia) using SYBR Green as intercalating fluorescent dye. The *NF2* gene, located on chromosome 22q12, was used as an internal reference locus.

Lymphocyte DNAs from 10 control subjects were used as reference normal samples for the optimization of experimental conditions, DNA derived from the Jurkat cell line was used as a homozygous deleted sample and the 1:1 mixture of Jurkat and wild-type DNA was used as a hemideleted sample.

All primer sequences (Tab. 1) were designed using the Primer 3 program (Rozen and Skaletsky). One μl of sample DNA (10 ng) was added to the PCR reaction mixture containing 2X SYBR Green Master Mix and 200 nmol/l

forward and reverse primers in a final volume of 10 µl. All analyses were performed in triplicate. The PCR amplification profile was as follows: initial denaturation at 95°C for 10min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing for 40 s and extension at 72°C for 20 s. Detection of the fluorescent product was carried out during the annealing period. Data analysis was performed using the comparative Delta Delta Ct (ΔΔCt) method (Livak and Schmittgen) The relative gene copy number was calculated using the expression $2^{-\Delta\Delta Ct}$, in order to establish the range of values for each group, standard deviations (SD) from the mean value were also calculated.

Microsatellite Analysis

Ten individuals belonging to 7 families segregating two recurrent *CDKN2A* mutations, c.67G>A (p.G23S) (n = 3) and c.[339G>C; 340C>T] (p.P114S) (n = 4) and 41 controls, were genotyped at eight simple tandem repeat markers located within or adjacent to *CDKN2A*. The following microsatellites, listed in order from centromere to telomere, were analyzed: *D9S171*, *D9S1604*, *D9S1748*, *D9S942*, *D9S974*, *D9S1749*, *D9S736*, and *IFNA* (Fig. 2). D*9S1749* and *D9S1604* flank the entire *CDKN2A* locus. For each primer pair, one oligonucleotide was labeled with a 50 fluorescent amidite (60 FAM, HEX or TET). Amplification was performed under standard PCR conditions using Taq Gold (Applied Biosystems, Foster City, California). PCR products were run on an ABI Prism 310 (Applied Biosystems) and analyzed by GeneScan (Applied Biosystems) software.

Fig.2 Schematic representation of microsatellite markers at the 9p21 locus.

Point mutation analysis

A total of 74 familial melanoma and multiple primary melanoma patients from unrelated kindreds have been screened for the presence of *CDKN2A* constitutional point mutations (Fig. 1).

Analysis of the entire coding sequence and intron-exon borders of the *CDKN2A* gene revealed the presence of 8 different point mutations, all located in exons 1α and 2, in 13 out of 74 probands (17,6%). Tab.2 summarizes the types of alterations and the number of families in which each single mutation was identified. Six of the eight mutations were observed only once, while the other two were seen in more than one family.

p16INK4A aa change	Type	N° Fam.
p.T18_A19del	small Del	1
p.G23S	Missense	3
p.R24P	Missense	1
p.A68L	Missense	1
p.G101W	Missense	1
p.L113L;P114S	Missense	4
p.R58X	Nonsense	1
p.E27X	Nonsense	1

Tab.2 Mutations detected and number of families in which the mutation has been found.

The p.E27X mutation (c.79G>T) in exon 1α was detected in a family with four CMM cases and presence of MPMs. This mutation generates a premature stop codon, leading to dramatically reduced protein levels of p16 and leaving p14ARF unaltered.

The small deletion c.52_57delACGGCC (p.T18_A19del), was identified in a familial melanoma patient affected also by pancreatic cancer. This small deletion has not been previously reported to the best of our knowledge; it segregated with the disease phenotype in the family, since it was present also in the nephew affected with CMM.

The other nonsense mutation (p.G101W) and three of the five missense mutations (p.R24P, p.R58X, p.A68L) detected have been previously described as being involved in melanoma susceptibility. The two additional recurrent missense mutations were investigated more thoroughly, as detailed hereafter.

Founder effect analysis

We identified two recurrent *CDKN2A* germline mutations in melanoma-prone families and MPM cases with positive family history.

One of these recurrent mutations (c.67G>A) had never been described and was found in patients from three different families (MF1, MF6, and MF17) in which there were ≥ 3 individuals who developed melanoma. In MF1 and MF6

families, multiple members were available for study (Fig. 4, 5, 6). This mutation converts a glycine to serine at codon 23 (p.G23S). It was present in all individuals affected with CMM in these families, whereas it was not detected in 100 cancer free controls originary from Tuscany with negative family history for CMM.

The second recurrent mutation was identified in patients from four different CMM families: three were MPM cases with positive family history of melanoma (MM14_MF, MM7_MF, MM22_MF), and the fourth was a familial melanoma case (MF33) without MPM cases. In MF33 and MM7_MF, two unaffected members were available for haplotype investigations, but none of the families had available affected members (Fig. 7, 8, 9, 10). The mutant allele contained two tandem sequence changes, c.(339G>C;340C>T), p.(L113L;P114S), causing a single nonsynonymous amino acid change (Fig. 3) Sequence analysis performed in the members of the MF33 family revealed the presence of the mutation in the non affected son of the proband at the age of 36 years .

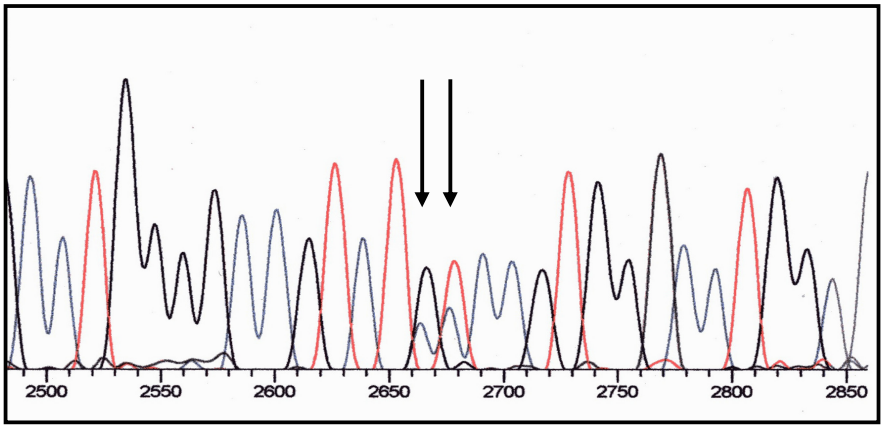

Fig. 3 Sequence of exon 2 showing the tandem mutation p.(L113L;P114S)

Although no apparent relationship between the families segregating the recurrent mutations could be found upon pedigree reconstruction, they all originated from the same geographic area in Tuscany. Therefore, to verify whether the over-representation of these mutations in the sample investigated could be due to a founder effect, we performed haplotype analysis on probands who were carriers as well as on available family members and on 41 controls originally from Tuscany without a family history of CMM. To this purpose we used eight microsatellite markers in the 9p21 region containing the *CDKN2A* locus (Fig. 2).

Analysis of *CDKN2A*-linked markers in p.G23S carriers and family members showed that a common haplotype, spanning a region of about 3 Mb, was present in all heterozygotes and cosegregated with the mutation in families MF1 and MF6 (Fig. 4, 5). No recombination events were observed among the markers investigated. The marker genotypes of the single individual available

for analysis from family MF17 were compatible with the common haplotype identified in the other two families (Fig. 6).

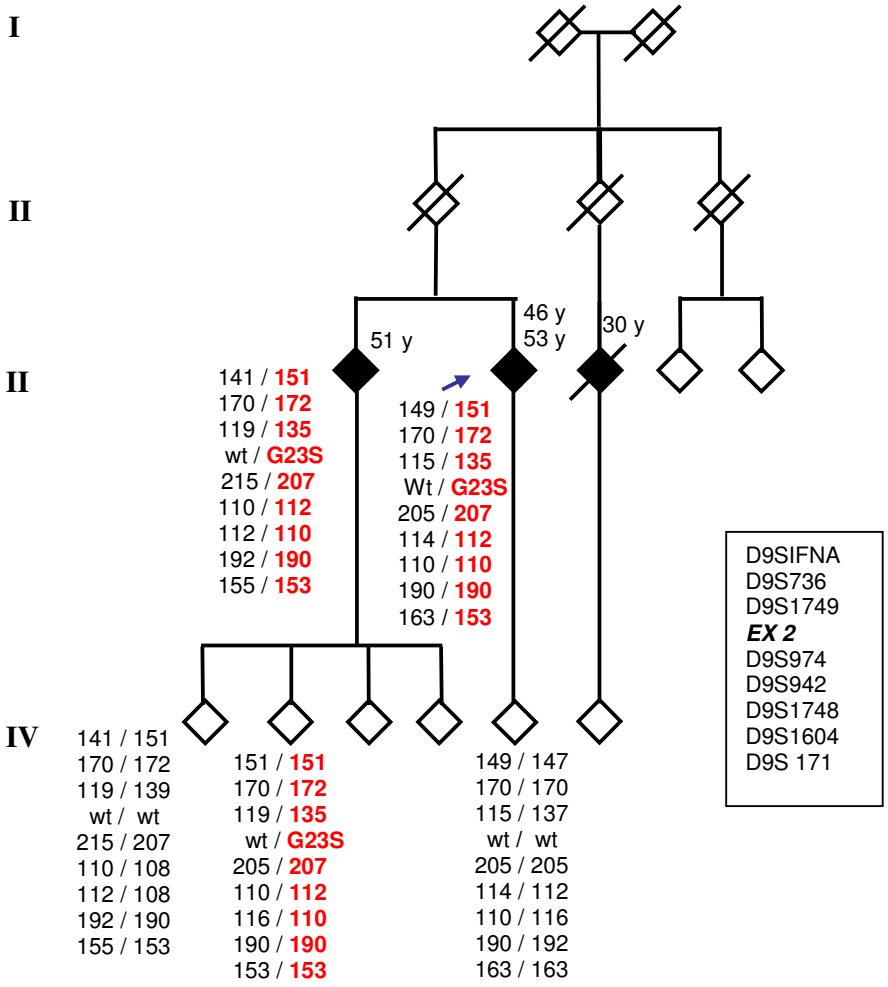

Fig. 4 Pedigree and haplotype analysis of family MF1_MM

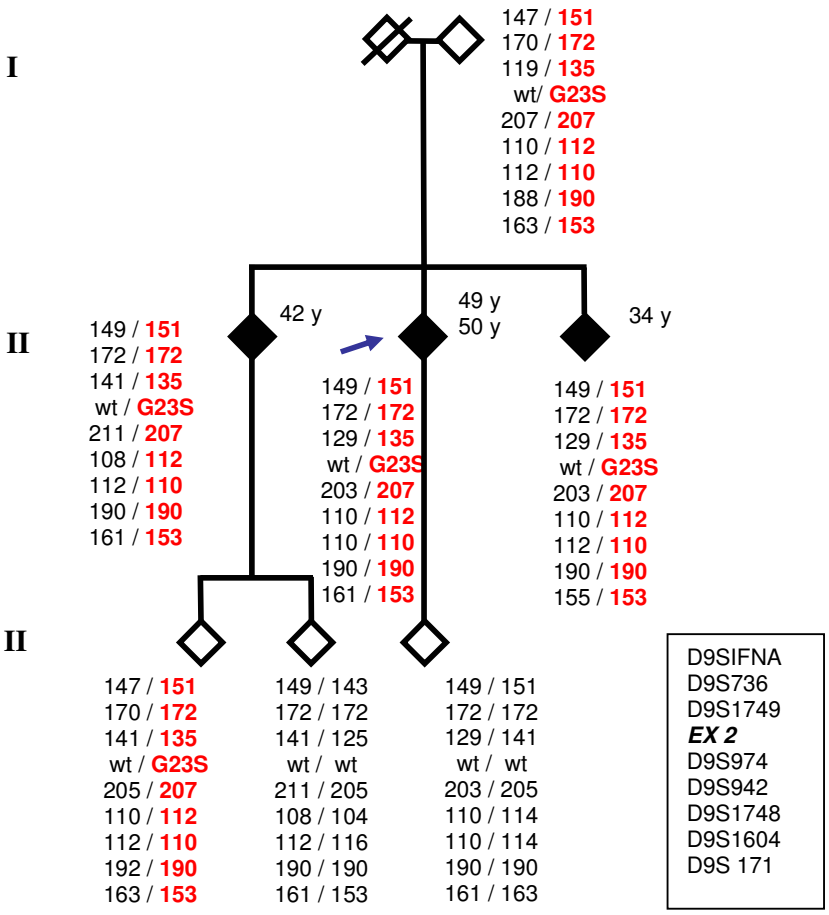

Fig. 5 Pedigree and haplotype analysis of family MF6_MM

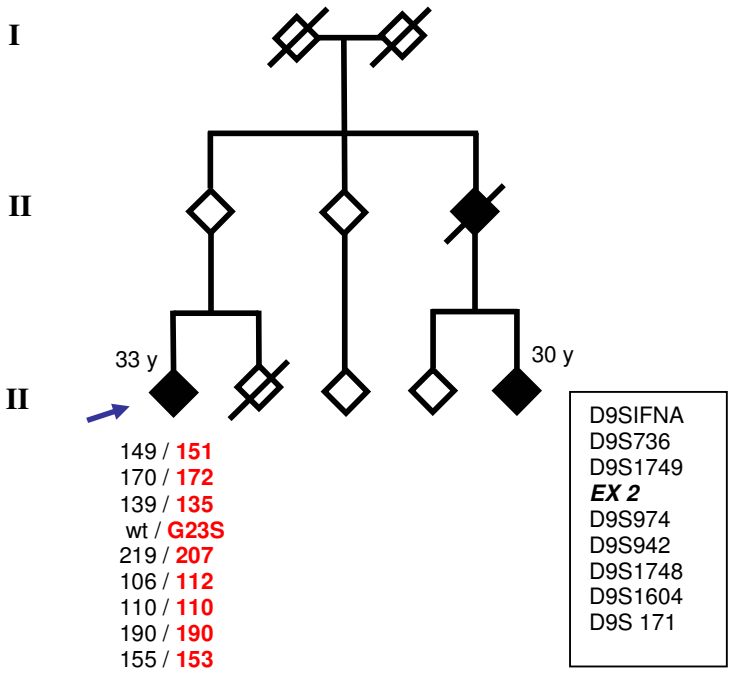

Fig. 6 Pedigree and haplotype analysis of family MF17

Analysis of microsatellite markers in p.L113L;p.P114S heterozygotes from families MM7_MF and MF33 and their non affected relatives allowed us to establish chromosome phase and to infer a common haplotype, that was shared also by the MF33 mutation-positive proband's son (Fig. 7, 8). Conversely, the combination of alleles present in the common haplotype was absent in non-carrier family members and was not found in the 82 control chromosomes. A recombination event was observed between markers *D9S1604* and *D9S171* in the proband from family MF7_MM, and a discrepant allele, that may have

arisen through replication slippage, was detected at marker *D9S1749* in the proband from family MF14_MM.

Finally, in the two families with only one individual available for analysis, the observed genotypes were compatible with the shared haplotype identified in families with the same mutation (Fig. 9, 10).

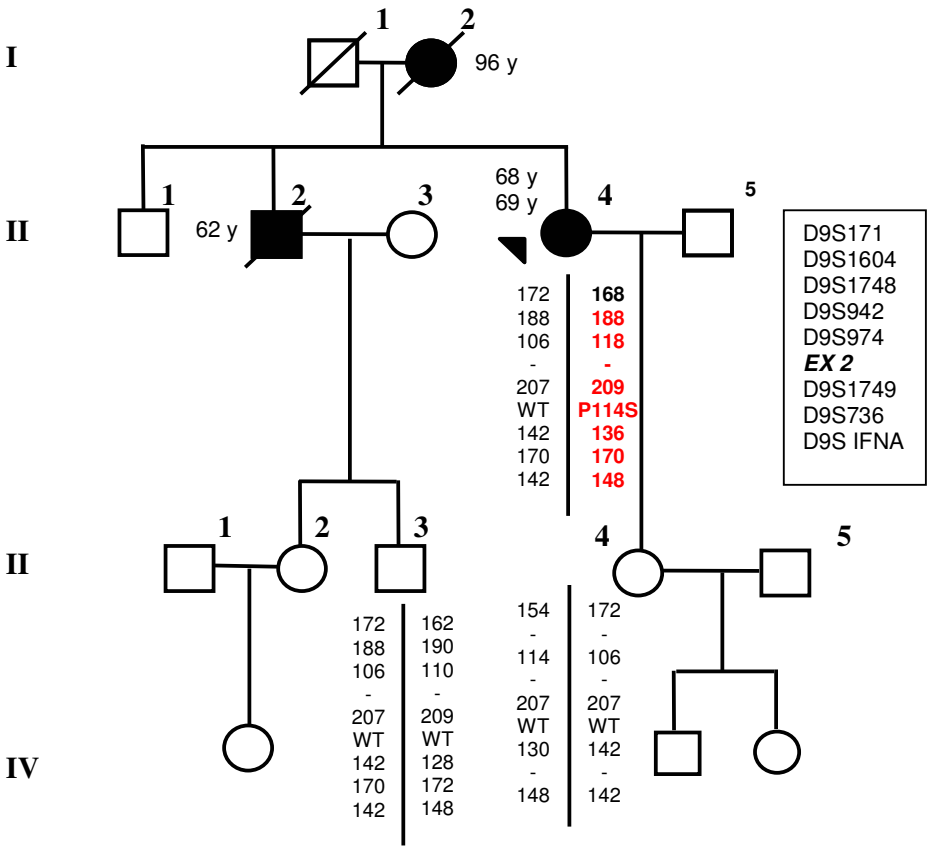

Fig. 7 Pedigree and haplotype analysis of family MM7_MF

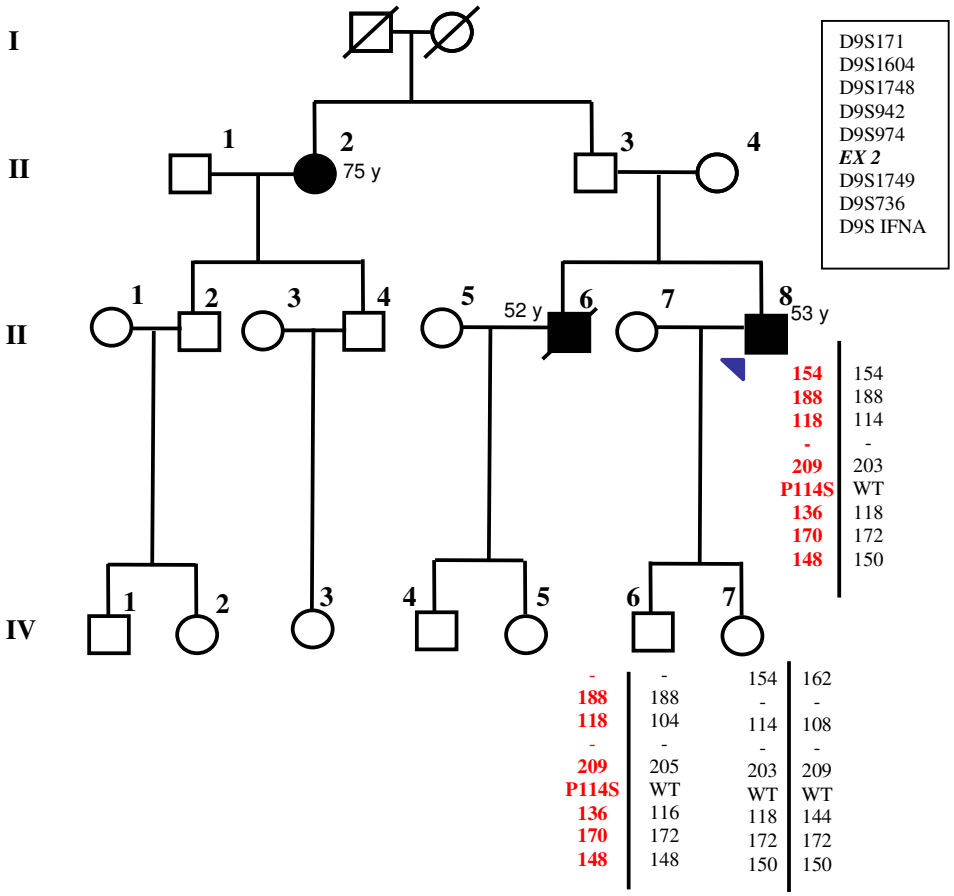

Fig. 8 Pedigree and haplotype analysis of family MF33

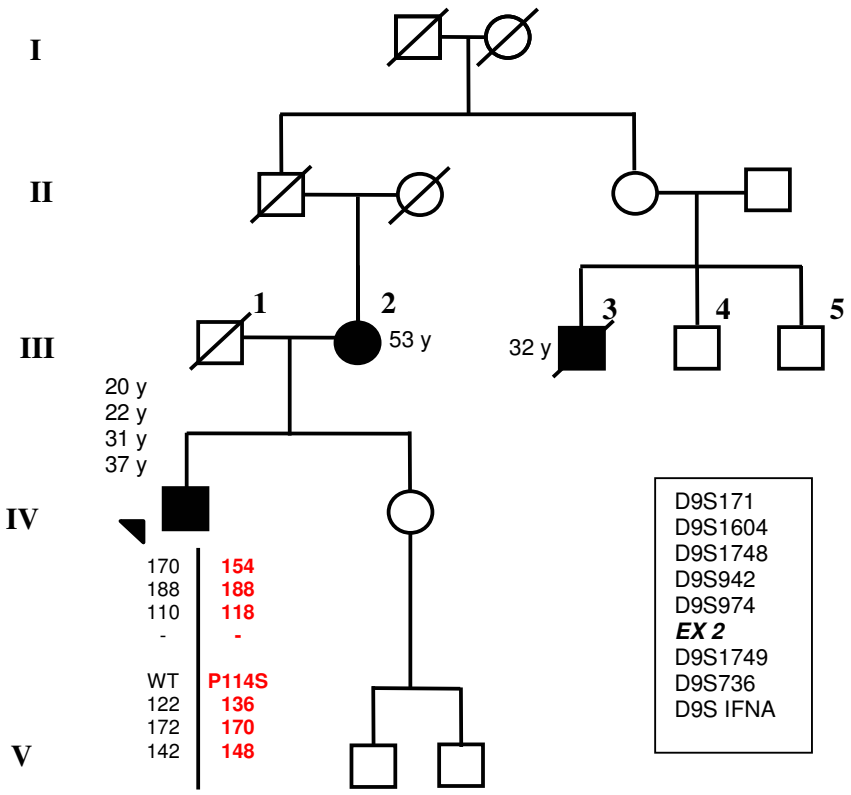

Fig. 9 Pedigree and haplotype analysis of family MM14_MF

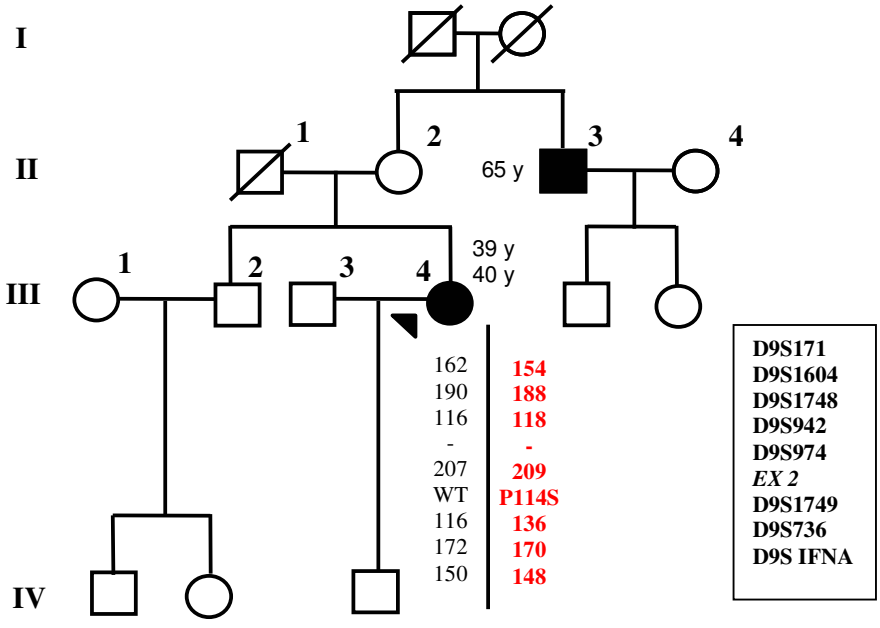

Fig. 10 Pedigree and haplotype analysis of family MM22_MF

The frequencies of alleles present in the common haplotype in the control population and in the melanoma patients are reported in Tab. 3.

χ^2 analysis showed a signifincant difference between frequencies of five of the seven markers analysed (P<0,01).

Marker	Common allele	Patients Frequency	Controls Frequency
D9S171	154	0,75	0,17
D9S1604	188	1	0,67
D9S1748	118	1	0,1
D9S942	-	-	-
D9S974	209	1	0,12
D9S1749	136	0,75	0,07
D9S736	170	1	0,35
D9SIFNA	148	1	0,6

Tab.3 Frequencies of alleles of the supposed common haplotype.

Genotype-phenotype correlations

Patients were divided into two subgroups based on clinical features: 49 patients were FCMM cases with or without a personal or family history of MPMs, while the remaining 25 probands were sporadic MPM cases without affected relatives. The frequency of mutation-positive families was related to clinical and geneticcharacteristics and was different in the two subgroups: all the mutations were identified in FCMM cases with or without MPMs (13/49: 26,5%). In the 29 melanoma-prone families with two affected members the

mutation frequency was 10,3% (n = 3) while in 20 families with at least 3 cases it was 50% (n = 10). The number of probands with a mutation was 5 among the 31 melanoma families without MPMs (16,1%), and 8 (44,4%) among 18 MPM patients with a positive family history; none of the sporadic MPM cases had *CDKN2A* mutations. Moreover, pancreatic cancer cases were reported in 5 of the 13 mutation-positive families.

Analysis of CDKN2A genomic rearrangements

Multiplex ligation-dependent probe amplification analysis

Preliminary experiments conducted on three control samples, on the Jurkat cell line and on a 1:1 mixture reproducing a monoallelic deletion of the full *CDNK2A* locus yielded results in line with the expected gene dosage, thus demonstrating the reliability of the MLPA approach (Fig. 11). In five out of 124 familial melanoma samples, values (0.47–0.57) in the range corresponding to a condition of hemizygous deletion were detected for the peak corresponding to the promoter region upstream of exon 1β. All samples were shown to contain two copies of other *CDKN2A* fragments and of surrounding regions investigated by the kit.

In order to verify whether the reduction in the peak area corresponding to the $CDKN2A^{ARF}$ promoter region observed by MLPA in five samples was due to a

genomic rearrangement or to variations within the sequence recognized by the MLPA probe, we performed direct sequencing of a 522 bp tract of the region upstream of exon 1β in these samples. A 6 bp deletion (c. – 1173_ – 1178del) located within the site of the probe used for hybridization was detected in all five samples. Segregation analysis was undertaken in a CMM pedigree: the proband's unaffected father, considered as an obligate carrier since his brother (the proband's uncle) was affected, did not have the variant identified in his affected son. The 6 bp deletion was also observed in a heterozygous state in three of 96 control samples.

Real-time gene dosage

In order to assess the accuracy of real-time quantitative PCR to reveal dosage differences at the *CDKN2A* locus, we first tested DNA from 10 control samples, from the Jurkat cell line and from one artificial hemizygous sample. These results were useful to define the ranges of values corresponding to wild type, hemideleted and homodeleted samples (Tab. 2). The results of real-time gene dosage confirmed the absence of large deletions in the *CDKN2A* coding regions in this familial CMM series.

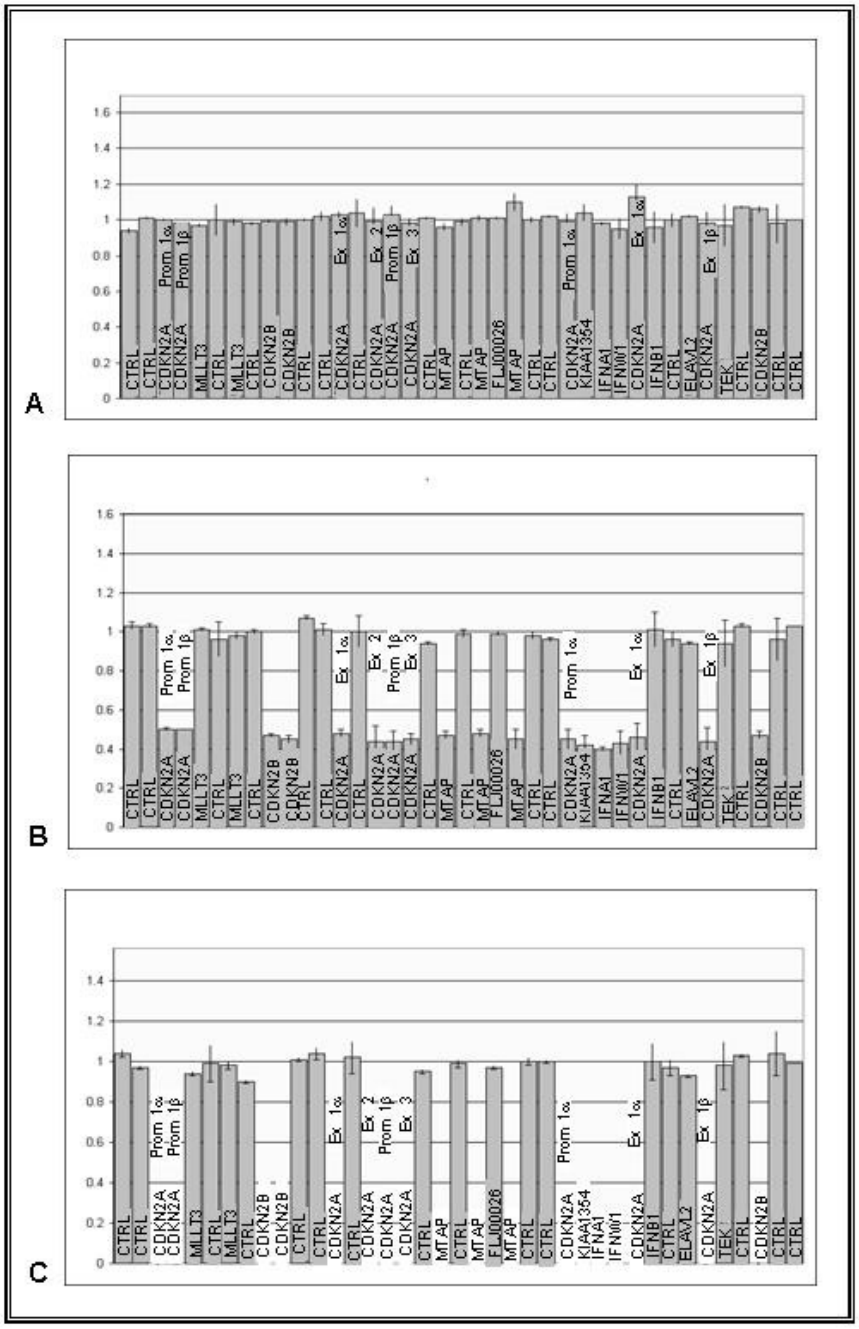

Fig.11 MLPA analysis of the CDKN2A region. (a) Wild-type control sample. (b) Hemideleted sample (Jurkat:control 1:1 mixture). (c) Jurkat DNA (with homozygous deletion). Symbols of investigated loci are shown on each bar (CTRL: control locus).

DISCUSSION AND CONCLUSIONS

CDKN2A is the most common high penetrance susceptibility gene identified to date in melanoma-prone families (Kamb et al., 1994; Hussussian et al., 1994). Mutations in the other known high-risk melanoma susceptibility gene, *CDK4*, are very rare (de Snoo et al., 2005).

On average the *CDKN2A* mutation detection rate in high risk melanoma families is about 20–40%, but it varies from 5% to 60% according to population characteristics and family selection criteria (Hayward et al., 2003, Leachman et al., 2009).

A recent Italian collaborative study, conducted on 208 Italian melanoma families with at least 2 affected relatives in the same branch of the family and MPM affected members, reported an overall mutation rate of 39% (Bruno et al., 2009).

Higher numbers of family members affected with CMM, earlier age at diagnosis, and the presence of multiple primary melanomas are all factors that strongly influence mutation detection rates regardless of the technical approach employed (Harland et al., 2008).

The aim of this study was to evaluate the contribution of *CDKN2A* mutations to the development of CMM, to assess mutation spectrum, and to evaluate the contribution of founder effects in the Tuscan population.

To this purpose, we have investigated 74 unrelated melanoma patients divided in three subgroups: FCMM cases, MPM cases with a positive family history and sporadic MPM patients. We detected 8 different point mutations in 13 probands (17,6%) by direct genomic sequencing of *CDKN2A* exons and intron borders.

Since the presence of large deletions or other genomic rearrangements in 9p21 might explain susceptibility in *CDKN2A* point mutation-negative melanoma families we then undertook a comprehensive analysis to detect large constitutional deletions/duplications.

MLPA was used as the screening method to investigate the whole series of 124 samples. It allows analysis of the whole region encompassing the *CDKN2A* locus and neighbouring 9p21 regions, but it may occasionally yield false positives due to the presence of sequence variants located within the regions covered by the hybridization probes (Knappskog et al., 2006). We therefore decided to confirm the normal MLPA results obtained on a subset of samples, for which sufficient DNA was available, by means of Real-Time quantitative PCR, using primers for different portions of the same exons. In five samples, MLPA analysis showed a reduction of the peak area corresponding to the promoter region of exon 1β. Direct sequencing revealed in all these cases the presence of a 6 bp deletion within the target sequence hybridizing to the MLPA probe. The same deletion was found in five probands and in three of 96 healthy controls, with allelic frequencies of 0.02 and 0.015 in the familial CMM and control population, respectively. In addition, it did not segregate with the disease phenotype in one melanoma family investigated. Therefore, we conclude that this deletion represents a low frequency polymorphism probably

not implicated in disease predisposition, although its involvement as a low-moderate risk factor for CMM cannot be completely ruled out. This issue would need investigation of larger familial CMM series and/or case-control studies.

No gross rearrangements in the *CDKN2A* coding regions and in the p16-specific promoter were detected in this large sample series; this result was confirmed with both methods in 53 samples, indicating that genomic alterations of the *CDKN2A* region not detectable by MLPA should be quite rare.

Overall, we found that large rearrangements of the *CDKN2A* locus are infrequently involved in the development of familial CMM in the Italian population. Therefore, routine search for these rearrangements in *CDKN2A* and *CDK4*-mutation negative CMM families does not seem to be warranted, although it would be reasonable to pursue it in selected cases with very strong family history and/or showing linkage to 9p21.

Overall, the frequency of *CDKN2A* mutations in our series was 17,6% (13/74). All alterations have been identified in familial melanoma patients with or without MPMs, and the frequency in this group (13/49: 26,5%) becomes closer to the value reported in the recent Italian collaborative study (Bruno et al., 2009). The mutation frequency showed a positive relationship with the number of affected members in the family: it was 10,3% (3/29) in pedigrees with 2 CMM cases versus 50% (10/20) in those with ≥3 cases. A higher *CDKN2A* mutation probability was associated also with the presence of individuals affected with MPM: 8/18 mutation-posititve cases were familial melanoma

probands with a personal or family history of MPMs (44,4%), while mutations were identified in 5 of the 31 (16,1%) FCMM probands with no MPMs.

Pancreatic cancer was also found to be associated with the presence of *CDKN2A* mutations. In 5 of the 13 (38,5%) mutation-positive pedigrees there were one or more family members affected with pancreatic carcinoma. Overall, these results show that a higher number of CMM affected members per family, the presence of MPM, and the combination of melanoma and pancreatic cancer are associated with a higher probability to detect *CDKN2A* mutations. These findings are consistent with those observed in larger Italian and European studies (Goldstein et al., 2007; Bruno et al., 2009).

The *CDKN2A* mutation spectrum observed in our series was characterized by a preponderance of single missense aminoacid substitutions (5/8; 62.5%); nonsense mutations (n= 2) accounted for 25% of the mutations, and the remaining mutation (12,5%) was a small in frame deletion. The distribution of *CDKN2A* mutation types is consistent with that observed in the Human Gene Mutation Database and in a previously European study (Goldstein et al., 2006).

Six of the eight mutations (p.R24P, p.E27X, p.R58X, p.A68L, p.G101W and p.[L113L;P114S]) detected in this series have been previously reported in familial melanoma cases, while the remaining two (p.T18_A19del; p.G23S) were novel mutations.

The functional effects of 5 of the sequence variants identified in this study are well established, either because they are truncating mutations or based on the

results of functional studies and segregation analysis (Hussussian et al., 1994; Fargnoli et al., 1998; Soufir et al., 1998; Ghiorzo et al., 2006; Kannengiesser et al., 2009).

The small deletion c.52_57delACGGCC (p.T18_A19del) was a novel variation detected in a familial melanoma case who was also affected with pancreatic cancer. It segregates with the disease phenotype in the family. A small duplication of 6 bp involving the same nucleotides (p.T18_A19dup) had been described in a pancreatic carcinoma cell line, in a melanoma family and in a MPM case (Naumann et al., 1996; Soufir et al., 1998). The two aminoacid residues involved in these alterations are located in the first ankyrin repeat that is involved in CDK6/CDK4 binding (Russo et al., 1998). Functional *in vitro* evaluation has shown that the protein carrying the duplication is unable to interact with CDK4, indicating that it is a loss of function mutation (Kannengiesser et al., 2009). Based on these considerations, it is highly likely that the in frame deletion identified in family MF37 is also pathogenic.

The novel p.G23S mutation was found in three melanoma families, two of which had one or more MPM affected members. Three other missense mutations affecting the same codon (p.G23C, p.G23D and p.G23R) had been previously described in melanoma-prone families (Soufir et al., 1998; Fargnoli et al., 1998; Goldstein et al., 2006). This amino acid residue is highly conserved (Goldstein et al., 2006). Assessment of its putative effects on protein structure by means of the Polyphen algorithm predicts a benign nature. Although no functional data are available on the effects of this mutation at the cellular and biochemical level, several lines of evidence indicate that the p.G23S change is involved in melanoma predisposition: evaluation of multiple

affected members in two of the three families from this study showed that it cosegregates with the CMM phenotype; it was not detected in a control population of the same geographic origin; and functional assays have demonstrated that mutations affecting the consensus amino acid residues in the first ankyrinic domain of p16INK4A, where G23 is located, reduce its binding and inhibitory activities (Ruas et al., 1999; Kannengiesser et al., 2009).

The p.(Leu113Leu;Pro114Ser) mutation was firstly described in French melanoma-prone families (Kannengiesser et al., 2007), but the functional consequences of this mutation have recently been investigated (Kannengiesser et al., 2009). By considering genetic information (cosegregation within families and frequency in a control population), the predicted impact of the variant on the protein structure (tertiary structure modeling, phylogenic conservation, and functional impact predictions *in silico*), its ability to interact with CDK4 and impede cell proliferation in experimental settings, these authors concluded that this tandem variant can be classified as loss of function mutation.

Overall, five of the eight alterations identified in this series represent founder mutations.

p.R24P is one of the most recurrent *CDKN2A* mutations observed in Australian and British melanoma families (Goldstein et al., 2006).

The p.E27X-positive family enrolled in this study was subsequently included in a larger study coordinated by the Department of Oncology, Biology and Genetics of the University of Genoa, where it was shown that it is one of the most frequent *CDKN2A* founder mutation identified to date in Italian melanoma families (Ghiorzo et al., 2006).

The missense mutation p.G101W identified in a familial melanoma patient in this series is the most common mutation observed to date in families worldwide (Goldstein et al., 2006).

The two recurrent missense variations, p.G23S and p.(L113L;P114S), were identified in melanoma-prone families and MPM cases with positive family history.

All p.G23S-positive families were originary from Tuscany, showing that this mutation is an important cause of hereditary melanoma in this region. Although these families were apparently unrelated, the results of haplotype analysis strongly indicate that the mutation is derived from a common ancestor.

The p.(L113L;P114S) mutation was firstly described as a founder germline mutation in a French study, where it was identified in three melanoma-prone families and one sporadic MPM case (Kannengiesser et al., 2007).

In our series, this mutation was detected in one familial melanoma patient and in three MPM probands with a positive family history. Haplotype analysis of the four probands, of the available non affected relatives and of 82 control chromosomes, allowed us to identify the most probable common haplotype. Allelic frequencies at *CDKN2A*-linked microsatellite markers were significantly different from those observed in control chromosomes as assessed by means of χ^2 analysis ($P<0.01$).

The analysis of founder mutations can potentially be of great interest for the evaluation of the role of other genetic and environmental modifiers in determining tumor developmente risk in families segregating a “major” predisposing mutation. As modifying genes, environmental sun exposure and

sun behavior attitudes vary across countries, Goldstein et al. (2007) analysed 79 p.G101W heterozygotes from 4 distinct countries (3 European and 1 in North America) to study the role of *MC1R* as a modifier gene and phenotypic features in the development of melanoma on a unique *CDKN2A* genetic background defined by a founder effect. Their study provided evidence that nonsynonymous variants in *MC1R* can modulate risk of melanoma development in individuals who are heterozygotes for *CDKN2A* mutations.

The two mutations detected in our series for which we documented a founder effect represent further additions to the group of *CDKN2A* founder mutations described worldwide. In particular in the Italian population the p.G101W mutation accounts for approximately 60% of the mutations detected, and another 23% of the mutations identified were known as possible founder mutations, or were recurrent (Bruno et al., 2009). Thus, founder or recurrent mutations seem to underlie susceptibility in the majority of familial melanoma cases in Italy, and may explain in part the relatively high frequency (3/29; 10,3%) of *CDKN2A* mutations in families with only 2 CMM cases in this series. Indeed, *CDKN2A* mutation detection rates remain lower than 10% in high CMM incidence areas, such as Australia, until the number of affected relatives in the family is al least 5.

These findings can be relevant in the framework of genetic counseling and testing for melanoma-prone families and provide the bases for further evaluation of modifier factors in genetically homogeneous baackgrounds.

REFERNCES

Antoniou A, Pharoah PD, Narod S, Risch HA, Eyfjord JE, Hopper JL, Loman N, Olsson H, Johannsson O, Borg A, Pasini B, Radice P, Manoukian S, Eccles DM, Tang N, Olah E, Anton-Culver H, Warner E, Lubinski J, Gronwald J, Gorski B, Tulinius H, Thorlacius S, Eerola H, Nevanlinna H, Syrjakoski K, Kallioniemi OP, Thompson D, Evans C, Peto J, Lalloo F, Evans DG, Easton DF. **2003**. Average risks of breast and ovarian cancer associated with BRCA1 or BRCA2 mutations detected in case Series unselected for family history: a combined analysis of 22 studies. Am J Hum Genet 72:1117–1130.

Armstrong BK, English DR. **1996**. Cutaneous malignant melanoma. In: D. Shottenfield, Fraumeni JF, Jr, editors. Cancer epidemiology and prevention. New York: Oxford University Press. p. 1312.

Bahuau M, Vidaud D, Jenkins RB, Bieche I, Kimmel DW, Assouline B, et al. **1998**. Germ-line deletion involving the INK4 locus in familial proneness to melanoma and nervous system tumors. Cancer Res; 58:2298–2303.

Balzi D, Bidoli E, Franceschi S, Arniani S, Pisani P, Geddes M, Associazione Italiana Registri Tumori. **1997**. Estimates of cancer incidence and mortality in Italian regions. Aviano, Italia: Centro di Riferimento Oncologico di Aviano. p 48-49.

Begg CB, Orlow I, Hummer AJ, Armstrong BK, Kricker A, Marrett LD, Millikan RC, Gruber SB, Anton-Culver H, Zanetti R, Gallagher RP, Dwyer T, Rebbeck TR, Mitra N, Busam K, From L, Berwick M, Genes Environment and Melanoma Study Group. **2005**. Lifetime risk of melanoma in CDKN2A mutation carriers in a population-based sample. J Natl Cancer Inst 97:1507–1515.

Bennett DC, **2008**. How to make a melanoma: what do we know of the primary clonal events? Pigment Cell Melanoma Res 21:27–38.

Bishop JA, Harland M, Bennett DC, Bataille V, Goldstein AM, Tucker MA, Ponder BA, Cuzick J, Selby P, Bishop DT. **1999.** Mutation testing in

melanoma families: INK4A, CDK4 and INK4D. Br J Cancer. 80 (1-2):295-300.

Bishop DT, Demenais F, Goldstein AM, Bergman W, Bishop JN, Bressac-de Paillerets B, et al. **2002.** Geographical variation in the penetrance of CDKN2A mutations for melanoma. J Natl Cancer Inst. 94:894-903.

Borg A, Johannsson U, Johannsson O, Häkansson S, Westerdahl J, Mäsbäck A, Olsson H, Ingvar C. **1996** Novel germline p16 mutation in familial malignant melanoma in southern Sweden. Cancer Res. 56(11):2497-500.

Box NF, Duffy DL, Chen W, Stark M, Martin NG, Sturm RA, Hayward NK. **2001.** MC1R genotype modifies risk of melanoma in families segregating CDKN2A mutations. Am J Hum Genet. 69:765–4.

Bruno W, Ghiorzo P, Battistuzzi L, Ascierto PA, Barile M, Gargiulo S, Gensini F, Gliori S, Guida M, Lombardo M, Manoukian S, Menin C, Nasti S, Origone P, Pasini B, Pastorino L, Peissel B, Pizzichetta MA, Queirolo P, Rodolfo M, Romanini A, Scaini MC, Testori A, Tibiletti MG, Turchetti D, Leachman SA, Bianchi Scarrà G; IMI, Italian Melanoma Intergroup. **2009**. Clinical genetic testing for familial melanoma in Italy: a cooperative study. J Am Acad Dermatol. 61(5):775-82.

Byeon IJ, Li J, Ericson K, Selby TL, Tevelev A, Kim HJ, O'Maille P, Tsai MD. **1998**. Tumor suppressor p16INK4A: determination of solution structure and analyses of its interaction with cyclin-dependent kinase 4. Mol Cell 1:421–431.

Chaudru V, Laud K, Avril M-F, Miniere A, Chompret A, Bressac-de Paillerets B, Demenais Fandthe French Familial Melanoma Study Group. **2005.** Melanocortin-1 (MC1R) gene variants and dysplastic nevi modify penetrance of CDKN2A mutations in French melanoma-prone pedigrees. Cancer Epidemiol Biomarkers Prev. 14:2384–90.

Ciotti P, Struewing JP, Mantelli M, et al. **2000**. A single genetic origin for the G101W CDKN2A mutation in 20 melanoma-prone families. Am J Hum Genet. 67: 311–9.

Dahl C, Guldberg P, **2007**. The genome and epigenome of malignant melanoma. APMIS 115:1160–1175

de Snoo FA, Hayward NK, **2005.** Cutaneous melanoma susceptibility and progression genes. Cancer Lett 230:153–186

Debniak T, Go´ rski B, Scott RJ, Cybulski C, Medrek K, Zowocka E, et al. **2004.** Germline mutation and large deletion analysis of the CDKN2A and ARF genes in families with multiple melanoma or an aggregation of malignant melanoma and breast cancer. Int J Cancer. 110:558–562.

Della Torre G, Pasini B, Frigerio S, Donghi R, Rovini D, Delia D, et al. **2001** CDKN2A and CDK4 mutation analysis in Italian melanoma-prone families: functional characterization of a novel CDKN2A germ line mutation. Br J Cancer 85: 836-44.

Erlandson A, Appelqvist F, Wennberg AM, Holm J, Enerback C **2007** Novel CDKN2A mutations detected in Western Swedish families with hereditary malignant melanoma. J Invest Dermatol 127: 1465– 1467.

Fargnoli MC, Chimenti S, Keller G, Soyer HP, Dal Pozzo V, Höfler H, Peris K. **1998** CDKN2a/p16INK4a mutations and lack of p19ARF involvement in familial melanoma kindreds. J Invest Dermatol. 111(6):1202-6.

Fargnoli MC, Altobelli E, Keller G, Chimenti S, Hofler H, Peris K. **2006** Contribution of melanocortin-1 receptor gene variants to sporadic cutaneous melanoma risk in a population in central Italy: a case-control study. Melanoma Res 16:175–82.

Ferrone CR, Ben Porat L, Panageas KS, Berwick M, Halpern AC, Patel A, et al. **2005** Clinicopathological features of and risk factors for multiple primary melanomas. JAMA 294:1647-54.

Fitzgerald MG, Harkin DP, Silva-Arrieta S, MacDonald DJ, Lucchina LC, Unsal H, O'Neill E, Koh J, Finkelstein DM, Isselbacher KJ, Sober AJ, Haber DA. **1996**. Prevalence of germline mutations in p16, p19ARF, and CDK4 in familial melanoma: Analysis of a clinic-based population. Proc Natl Acad Sci USA 93:8541–8545.

Forbes SA, Bhamra G, Bamford S, Dawson E, Kok C, Clements J, Menzies A, Teague JW, Futreal PA, Stratton MR **2008** The Catalogue of Somatic Mutations in Cancer (COSMIC). Curr Protoc Hum Genet Chapter 10:Unit 10.11.

Ghiorzo P, Ciotti P, Mantelli M, Heouaine A, Queirolo P, Rainero ML, Ferrari C, Santi PL, De Marchi R, Farris A, Ajmar F, Bruzzi P, Bianchi-Scarrà G. **1999** Characterization of ligurian melanoma families and risk of occurrence of other neoplasia. Int J Cancer. 83(4):441-8.

Ghiorzo P, Gargiulo S, Pastorino L, Nasti S, Cusano R, Bruno W, Gliori S, Sertoli MR, Burroni A, Savarino V, Gensini F, Sestini R, Queirolo P, Goldstein AM, Scarrà GB. **2006** Impact of E27X, a novel CDKN2A germ line mutation, on p16 and p14ARF expression in Italian melanoma families displaying pancreatic cancer and neuroblastoma. Hum Mol Genet. 15(18):2682-9.

Goldstein AM, Tucker MA. **1997**. Screening for CDKN2A mutations in hereditary melanoma. J Natl Cancer Inst 89:676-678.

Goldstein AM, Landi MT, Tsang S, Fraser MC, Munroe DJ, Tucker MA. **2005** Association of MC1R variants and risk of melanoma in melanoma-prone families with CDKN2A mutations. Cancer Epidemiol Biomarkers Prev;14:2208–12.

Goldstein AM, Chan M, Harland M, Gillanders EM, Hayward NK, Avril MF, et al. **2006**. High-risk melanoma susceptibility genes and pancreatic cancer, neural system tumors, and uveal melanoma across GenoMEL. Cancer Res 66:9818-28.

Goldstein AM, Chan M, Harland M, Hayward NK, Demenais F, Bishop DT, Azizi E, Bergman W, Bianchi-Scarra G, Bruno W, Calista D, Albright LA, Chaudru V, Chompret A, Cuellar F, Elder DE, Ghiorzo P, Gillanders EM, Gruis NA, Hansson J, Hogg D, Holland EA, Kanetsky PA, Kefford RF, Landi MT, Lang J, Leachman SA, MacKie RM, Magnusson V, Mann GJ, Bishop JN, Palmer JM, Puig S, Puig-Butille JA, Stark M, Tsao H, Tucker MA, Whitaker L, Yakobson E; Lund Melanoma Study Group; Melanoma Genetics Consortium (GenoMEL). **2007** Features associated

with germline CDKN2A mutations: a GenoMEL study of melanoma-prone families from three continents. J Med Genet. 44(2):99-106.

Greene MH. **1999**. The genetics of hereditary melanoma and nevi. 1998 update. Cancer 86(Suppl 11):2464-2477.

Gruis NA, Sandkuijl LA, van der Velden PA, Bergman W, Frants RR. **1995a** CDKN2 explains part of the clinical phenotype in Dutch familial atypical multiple-mole melanoma (FAMMM) syndrome families. Melanoma Res. 5(3):169-77.

Gruis NA, van der Velden PA, Sandkuijl LA, et al. **1995b** Homozygotes for CDKN2 (p16) germline mutation in Dutch familial melanoma kindreds. Nat Genet;10: 351–3.

Harland M, Holland EA, Ghiorzo P, et al. **2000** Mutation screening of the CDKN2A promoter in melanoma families. Genes Chromosomes Cancer 28:45–57.

Harland M, Mistry S, Bishop DT, Bishop JA. **2001** A deep intronic mutation in CDKN2A is associated with disease in a subset of melanoma pedigrees. Hum Mol Genet; 10:2679–2686.

Harland M, Taylor CF, Bass S, Churchman M, Randerson-Moor JA, Holland EA, et al. **2005a** Intronic sequence variants of the CDKN2A gene in melanoma pedigrees. Genes Chromosomes Cancer 43:128–136.

Harland M, Taylor CF, Chambers PA, et al. **2005b** A mutation hotspot at the p14ARF splice site. Oncogene 24:4604–8.

Harland M, Goldstein AM, Kukalizch K, Taylor C, Hogg D, Puig S, Badenas C, Gruis N, ter Huurne J, Bergman W, Hayward NK, Stark M, Tsao H, Tucker MA, Landi MT, Scarra GB, Ghiorzo P, Kanetsky PA, Elder D, Mann GJ, Holland EA, Bishop DT, Bishop JN; GenoMEL, the Melanoma Genetics Consortium. **2008** A comparison of CDKN2A mutation detection within the Melanoma Genetics Consortium (GenoMEL). Eur J Cancer. 44(9):1269-74.

Hashemi J, Bendahl P-O, Sandberg T, et al. **2001** Haplotype analysis and age estimation of the 113insR CDKN2A founder mutation in Swedish melanoma families. Genes Chromosomes Cancer;31:107–16.

Hashemi J, Lindström MS, Asker C, Platz A, Hansson J, Wiman KG. **2002** A melanoma-predisposing germline CDKN2A mutation with functional significance for both p16 and p14ARF. Cancer Lett. 180(2):211-21.

Hayward NK **2003** Genetics of melanoma predisposition. Oncogene 22: 3053–3062.

Helsing P, Nymoen DA, Ariansen S, Steine SJ, Maehle L, Aamdal S, Langmark F, Loeb M, Akslen LA, Molven A, Andresen PA. **2008** Population-based prevalence of CDKN2A and CDK4 mutations in patients with multiple primary melanomas. Genes Chromosomes Cancer. 47(2):175-84.

Hewitt C, Lee Wu C, Evans G, Howell A, Elles RG, Jordan R, et al. **2002** Germline mutation of ARF in a melanoma kindred. Hum Mol Genet; 11:1273–1279.

Holland EA, Schmid H, Kefford RF, Mann GJ. **1999** CDKN2A (P16(INK4a)) and CDK4 mutation analysis in 131 Australian melanoma probands: effect of family history and multiple primary melanomas. Genes Chromosomes Cancer 25: 339-48.

Hussussian CJ, Struewing JP, Goldstein AM, Higgins PA, Ally DS, Sheahan MD, et al. **1994** Germline p16 mutations in familial melanoma. Nat Genet 8:15–21.

Kamb, A.; Gruis, N. A.; Weaver-Feldhaus, J.; Liu, Q.; Harshman, K.; Tavtigian, S. V.; Stockert, E.; Day, R. S., III; Johnson, B. E.; Skolnick, M. H. **1994** A cell cycle regulator potentially involved in genesis of many tumor types. Science 264: 436-440,.

Kannengiesser C, Dalle S, Leccia MT, Avril MF, Bonadona V, Chompret A, Lasset C, Leroux D, Thomas L, Lesueur F, Lenoir G, Sarasin A, Bressac-de Paillerets B. **2007** New founder germline mutations of CDKN2A in melanoma-prone families and multiple primary melanoma development in a patient receiving levodopa treatment. Genes Chromosomes Cancer. 46(8):751-60.

Kannengiesser C, Brookes S, del Arroyo AG, Pham D, Bombled J, Barrois M, Mauffret O, Avril MF, Chompret A, Lenoir GM, Sarasin A; French

Hereditary Melanoma Study Group, Peters G, Bressac-de Paillerets B. **2009** Functional, structural, and genetic evaluation of 20 CDKN2A germ line mutations identified in melanoma-prone families or patients. Hum Mutat. 30(4):564-74.

Kefford RF, Newton Bishop JA, Bergman W, Tucker MA. **1999**. Counseling and DNA testing for individuals perceived to be genetically predisposed to melanoma: a consensus statement of the Melanoma Genetics Consortium. J. Clin. Oncol. 17:3245–51.

Knappskog S, Geisler J, Arnesen T, Lillehaug JR, Lønning PE. **2006** A novel type of deletion in the CDKN2A gene identified in a melanoma-prone family. Genes Chromosomes Cancer 45:1155–1163.

Koh, J.; Enders, G. H.; Dynlacht, B. D.; Harlow, E. **1995** Tumour-derived p16 alleles encoding proteins defective in cell-cycle inhibition. Nature 375: 506-510.

Landi MT, Kanetsky PA, Tsang S, Gold B, Munroe D, Rebbeck T, Swoyer J, Ter-Minassian M, Hedayati M, Grossman L, Goldstein AM, Calista D, et al. **2005** MC1R, ASIP, and DNA repair in sporadic and familial melanoma in a Mediterranean population. J Natl Cancer Inst 97:998–1007.

Laud K, Marian C, Avril MF, Barrois M, Chompret A, Goldstein AM, et al. **2006** Comprehensive analysis of CDKN2A (p16INK4A/p14ARF) and CDKN2B genes in 53 melanoma index cases considered to be at heightened risk of melanoma. J Med Genet 43:39–47.

Leachman SA, Carucci J, Kohlmann W, Banks KC, Asgari MM, Bergman W, Bianchi-Scarrà G, Brentnall T, Bressac-de Paillerets B, Bruno W, Curiel-Lewandrowski C, de Snoo FA, Debniak T, Demierre MF, Elder D, Goldstein AM, Grant-Kels J, Halpern AC, Ingvar C, Kefford RF, Lang J, MacKie RM, Mann GJ, Mueller K, Newton-Bishop J, Olsson H, Petersen GM, Puig S, Rigel D, Swetter SM, Tucker MA, Yakobson E, Zitelli JA, Tsao H. **2009** Selection criteria for genetic assessment of patients with familial melanoma. J Am Acad Dermatol. 61(4):677.e1-14. Review.

Lilischkis R, Sarcevic B, Kennedy C, Warlters A, Sutherland RL. **1996**. Cancer associated missense and deletion mutations impair p16INK4 CDK inhibitory activity. Int J Cancer 66:249–254.

Liu L, Dilworth D, Gao L, Monzon J, Summers A, Lassam N, et al. **1999** Mutation of the CDKN2A 50 UTR creates an aberrant initiation codon and predisposes to melanoma. Nat Genet; 21:128–132.

Livak KJ, Schmittgen TD. **2001** Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)). Methods; 25:402–408.

Lukas, J.; Parry, D.; Aagaard, L.; Mann, D. J.; Bartkova, J.; Strauss, M.; Peters, G.; Bartek, J. **1995** Retinoblastoma-protein-dependent cell-cycle inhibition by the tumour suppressor p16. Nature 375: 503-506.

Majore S, Catricala C, Binni F, De Simone P, Eibenschutz L, Grammatico P. **2004**. CDKN2A: the IVS2-105A/G intronic mutation found in an Italian patient affected by eight primary melanomas. J Investig Dermatol 122:450–451.

Mantelli M, Barile M, Ciotti P, Ghiorzo P, Lantieri F, Pastorino L, et al. **2002** High prevalence of the G101W germline mutation in the CDKN2A (P16(ink4a)) gene in 62 Italian malignant melanoma families. Am J Med Genet;107: 214-21.

Mistry SH, Taylor C, Randerson-Moor JA, Harland M, Turner F, Barrett JH, et al. **2005** Prevalence of 9p21 deletions in UK melanoma families. Genes Chromosomes Cancer; 44:292–300.

Molven A, Grimstvedt MB, Steine SJ, et al. **2005** A large Norwegian family with inherited malignant melanoma, multiple atypical nevi, and CDK4 mutation. Genes Chromosomes Cancer;44:10–8.

Monzon J, Liu L, Brill H, Goldstein AM, Tucker MA, From L, et al. **1998** CDKN2A mutations in multiple primary melanomas. N Engl J Med;338:879-87.

Naumann,M., Savitskaia,N., Eilert,C., Schramm,A., Kalthoff H., Schmiegel,W. **1996** Frequent codeletion of p16/MTS1 and p15/MTS2 and genetic

alterations in p16/MTS1 in pancreatic tumors. Gastroenterology, 110, 1215–1224.

Ogawa S, Hirano N, Sato N, Takahashi T, Hangaishi A, Tanaka K, et al. **1994**. Homozygous loss of the cyclin-dependent kinase 4-inhibitor (p16) gene in human leukemias. Blood; 84:2431–2435.

Ohtani, N.; Zebedee, Z.; Huot, T. J. G.; Stinson, J. A.; Sugimoto, M.; Ohashi, Y.; Sharrocks, A. D.; Peters, G.; Hara, E. **2001** Opposing effects of Ets and Id proteins on p16(INK4A) expression during cellular senescence. Nature 409: 1067-1070,.

Pasmant E, Laurendeau I, Heron D, Vidaud M, Vidaud D, Bieche I **2007** Characterization of a germ-line deletion, including the entire INK4/ARF locus, in a melanoma-neural system tumor family: identification of ANRIL, an antisense noncoding RNA whose expression coclusters with ARF. Cancer Res 67: 3963–3969

Pastorino L, Cusano R, Bruno W, Lantieri F, Origone P, Barile M, Gliori S, Shepherd GA, Sturm RA, Bianchi-Scarra G. **2004** Novel MC1R variants in. Ligurian melanoma patients and controls. Hum Mutat;24:103.

Pastorino L, Bonelli L, Ghiorzo P, Queirolo P, Battistuzzi L, Balleari E, et al. **2008** CDKN2A mutations and MC1R variants in Italian patients with single or multiple primary melanoma. Pigment Cell Melanoma Res;21:700-9.

Pjanova D, Molven A, Akslen LA, Engele L, Streinerte B, Azarjana K, Heisele O. **2009** Identification of a CDK4 R24H mutation-positive melanoma family by analysis of early-onset melanoma patients in Latvia. Melanoma Res. 19(2):119-22.

Platz A, Hansson J, Mansson-Brahme E, et al. **1997** Screening of germline mutations in the CDKN2A and CDKN2B genes in Swedish families with hereditary cutaneous melanoma. J Natl Cancer Inst; 89:697–702.

Pollock PM, Spurr N, Bishop T, Newton-Bishop J, Gruis N, van der Velden PA, Goldstein AM, Tucker MA, Foulkes WD, Barnhill R, Haber D, Fountain J, Hayward NK. **1998**. Haplotype analysis of two recurrent

CDKN2A mutations in 10 melanoma families: evidence for common founders and independent mutations. Hum Mutat 11:424–431.

Pomerantz J, Schreiber-Agus N, Liegeois NJ, Silverman A, Alland L, Chin L, et al. **1998** The Ink4a tumor suppressor gene product, p19Arf, interacts with MDM2 and neutralizes MDM2's inhibition of p53. Cell;92:713–23.

Puig S, Malvehy J, Badenas C, Ruiz A, Jimenez D, Cuellar F, et al. **2005** Role of the CDKN2A locus in patients with multiple primary melanomas. J Clin Oncol;23:3043-51.

Randerson-Moor JA, Harland M,Williams S, Cuthbert-Heavens D, Sheridan E, Aveyard J, et al. **2001** A germline deletion of p14(ARF) but not CDKN2A in a melanoma-neural system tumour syndrome family. Hum Mol Genet; 10:55–62.

Rizos H, Darmanian AP, Holland EA, Mann GJ, Kefford RF. **2001a**. Mutations in the INK4a/ARF melanoma susceptibility locus functionally impair p14ARF. J Biol Chem 276:41424–41434.

Rizos H, Puig S, Badenas C, Malvehy J, Darmanian AP, Jime´ nez L, et al. **2001b** A melanoma-associated germline mutation in exon 1beta inactivates p14ARF. Oncogene; 20:5543–5547.

Rozen S, Skaletsky H. **2000** Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol; 132:365–386.

Ruas M, Brookes S, McDonald NQ, Peters G. **1999** Functional evaluation of tumour-specific variants of p16INK4a/CDKN2A: correlation with protein structure information. Oncogene. 18(39):5423-34.

Ruiz A, Puig S, Malvehy J, Lazaro C, Lynch M, Gimenez-Arnau AM, Puig L, Sanchez-Conejo J, Estivill X, Castel T. **1999**. CDKN2A mutations in Spanish cutaneous malignant melanoma families and patients with multiple melanomas and other neoplasia. J Med Genet 36:490-493.

Russo AA, Tong L, Lee JO, Jeffrey PD, Pavletich NP. **1998**. Structural basis for inhibition of the cyclin-dependent kinase Cdk6 by the tumour suppressor p16INK4a. Nature 395:237–243.

Serrano M, Hannon GJ, Beach D. **1993** A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. Nature;366:704–7.

Serrano M, Gomez-Lahoz E, DePinho RA, Beach D, Bar-Sagi D. **1995** Inhibition of ras-induced proliferation and cellular transformation by p16INK4. Science;267:249–52.

Soufir N, Avril MF, Chompret A, Demenais F, Bombled J, Spatz A, Stoppa-Lyonnet D, Bénard J, Bressac-de Paillerets B. **1998** Prevalence of p16 and CDK4 germline mutations in 48 melanoma-prone families in France. The French Familial Melanoma Study Group. Hum Mol Genet. 1998 7(2):209-16.

Van der Velden PA, Sandkuijl LA, Bergman W, Pavel S, van Mourik L, Frants RR, Gruis NA. **2001** Melanocortin-1 receptor variant R151C modifies melanoma risk in Dutch families with melanoma. Am J Hum Genet;69:774–9.

Yang R, Gombart AF, Serrano M, Koeffler HP. **1995**. Mutational effects on the p16INK4a tumor suppressor protein. Cancer Res 55:2503–2506.

Zhang B, Peng Z. **2000**. A minimum folding unit in the ankyrin repeat protein p16(INK4). J Mol Biol 299:1121–1132.

Zhang Y, Xiong Y, Yarbrough WG. **1998** ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. Cell;92:725–34.

Zhang, Y., Xiong, Y. **1999** Mutations in human ARF exon 2 disrupt its nucleolar localization and impair its ability to block nuclear export of MDM2 and p53. Molec. Cell 3: 579-591,.

Zuo L, Weger J, Yang Q, et al. **1996** Germline mutations in the p16INK4a binding domain of CDK4 in familial melanoma. Nat Genet;12:97–9.

PUBBLICATIONS

Vignoli M, Scaini MC, Ghiorzo P, Sestini R, Bruno W, Menin C, Gensini F, Piazzini M, Testori A, Manoukian S, Orlando C, D'Andrea E, Bianchi-Scarrà G, Genuardi M. Genomic rearrangements of the CDKN2A locus are infrequent in Italian malignant melanoma families without evidence of CDKN2A/CDK4 point mutations. Melanoma Res. 2008 Dec; 18(6):431-7.

Gensini F, Sestini R, Piazzini M, **Vignoli M**, Chiarugi A, Brandani P, Ghiorzo P, Salvini C, Borgognoni L, Palli D, Bianchi-Scarrà G, Carli P, Genuardi M. The p.G23S CDKN2A founder mutation in high-risk melanoma families from Central Italy. Melanoma Res. 2007 Dec; 17(6):387-92.

De Giorgi V, Salvini C, Sestini S, **Vignoli M**, Sestini R, Papi F, Lotti T. Triple synchronous cutaneous melanoma: a clinical, dermoscopic, and genetic case study. Dermatol Surg. 2007 Apr;33(4):488-91.