High Frequency of Multiple Melanomas and Breast and Pancreas Carcinomas in CDKN2A Mutation-Positive Melanoma Families

Åke Borg, Therese Sandberg, Kerstin Nilsson, Oskar Johannsson, Mikael Klinker, Anna Måsbäck, Johan Westerdahl, Håkan Olsson, Christian Ingvar

Background: **Inherited mutations in the CDKN2A tumor suppressor gene, which encodes the p16^{INK4a} protein, and in the cyclin-dependent kinase 4 (CDK4) gene confer susceptibility to cutaneous malignant melanoma. We analyzed families with two or more cases of melanoma for germline mutations in CDKN2A and CDK4 to elucidate the contribution of these gene defects to familial malignant melanoma and to the occurrence of other cancer types.** ***Methods:*** **The entire CDKN2A coding region and exon 2 of the CDK4 gene of an affected member of each of 52 families from southern Sweden with at least two cases of melanoma in first- or second-degree relatives were screened for mutations by use of polymerase chain reaction–single-strand conformation polymorphism analysis. Statistical tests were two-sided.** ***Results:*** **CDKN2A mutations were found in 10 (19%) of the 52 families. Nine families carried an identical alteration consisting of the insertion of arginine at position 113 of p16^{INK4a}, and one carried a missense mutation, in which the valine at position 115 was replaced with a glycine. The 113insArg mutant p16^{INK4a} was unable to bind cdk4 and cdk6 in an *in vitro* binding assay. Six of the 113insArg families had at least one member with multiple primary melanomas; the 113insArg families also had a high frequency of other malignancies—in particular, breast cancer (a total of eight cases compared with the expected 2.1; *P* = .0014) and pancreatic cancer (a total of six cases compared with the expected 0.16; *P*<.0001). Families with breast cancer also had a propensity for multiple melanomas in females, suggesting that a sex-dependent factor may modify the phenotypic expression of CDKN2A alterations.** ***Conclusions:*** **Our findings confirm that the majority of CDKN2A-associated melanoma families in Sweden are due to a single founder mutation. They also show that families with the CDKN2A 113insArg mutation have an increased risk not only of multiple melanomas and pancreatic carcinoma but also of breast cancer. [J Natl Cancer Inst 2000;92: 1260–6]**

Roughly 10% of cutaneous malignant melanoma occurs in a familial setting *(1).* Linkage analysis has implicated chromosomal region 9p21 as the site of a melanoma susceptibility gene *(2),* and the CDKN2A/MTS1/INK4A gene, which encodes the p16^{INK4a} tumor suppressor protein, represents the major candidate gene at this locus *(3–5).* The p16^{INK4a} protein binds to cdk4 (cyclin-dependent kinase 4) and cdk6, thereby blocking their ability to form catalytic complexes with cyclin D and preventing the subsequent phosphorylation of the retinoblastoma gene product, which is necessary for progression through the G_1 cell cycle checkpoint. The normal p16^{INK4a} protein thus maintains cell cycle arrest. A nonfunctional p16^{INK4a} protein has lost its regulatory capacity and cannot constrain cells from passing through the cell cycle *(6,7).* The p16^{INK4a} protein contains four ankyrin repeats that are involved in protein–protein interactions. Some residues in these repeats are part of the conserved consensus sequence, and mutations at these amino acids are thought to be essential to the function of the protein *(3).*

Germline mutations in the CDKN2A gene have been identified in the majority of 9p21-linked melanoma families, confirming this gene's role as the major melanoma susceptibility gene at this locus *(8–19).* The occurrence of 9p21 linkage without mutation in coding regions of CDKN2A may be explained by alterations in regulatory regions of the gene *(20),* large genomic deletions *(21),* or mutations in alternative genes in the vicinity of CDKN2A, such as the gene encoding p14^{ARF}, which includes the alternative exon 1β of the CDKN2A gene and an alternative reading frame within exon 2 of CDKN2A *(22,23),* or the CDKN2B gene, which encodes the p15^{INK4b} protein *(4).* Further evidence that impaired p16^{INK4a}-mediated cell cycle regulation may lead to melanoma development comes from the finding that a germline CDK4 mutation identified in a melanoma kindred abolishes the binding of cdk4 to p16^{INK4a} but preserves its ability to interact with cyclin D and its catalytic activity *(24).*

In previous investigations of Swedish families with malignant melanoma, germline CDKN2A mutations were observed in two of 10 families *(14)* and in 7.8% of 64 kindreds with two or more first degree relatives with malignant melanoma *(15).* The germline mutation 113insArg was identified in both investigations. This study was performed to estimate the contribution of CDKN2A mutations to melanoma and other cancers in Swedish melanoma families. We screened affected members of 52 families for mutations in the coding region of the CDKN2A gene for a specific mutation in the promoter region of the CDKN2A gene and for mutations in the region of CDK4 encoding the p16^{INK4a}-binding motif. We also assayed the *in vitro* cdk4/cdk6-binding activity of the forms of p16^{INK4a} encoded by the mutant alleles of CDKN2A identified in our study. Finally, we examined the cosegregation of the 113insArg mutation with other tumor types.

Materials and Methods

Families

A total of 52 families with two or more cases of malignant melanoma in first- or second-degree relatives, meeting the criteria of possible disease inheritance, were included in the study. Ten of these have been described earlier *(14);* they represent consecutive case patients treated at the Department of Surgery, Lund, Sweden, who reported a family history of melanoma. An additional 35 families were obtained from a population-based, case–control study of constitutional risk factors for malignant melanoma in southern Sweden, representing case patients reporting a positive family history of malignant melanoma. Four families were recruited through a specialized melanoma clinic at the Department of Dermatology, University Hospital, Lund. The re-

Affiliations of authors: Å. Borg, T. Sandberg, O. Johannsson, H. Olsson (Department of Oncology), K. Nilsson, J. Westerdahl, C. Ingvar (Department of Surgery), M. Klinker (Department of Dermatology), A. Måsbäck (Department of Pathology), University Hospital, Lund, Sweden.

Correspondence to: Åke Borg, Ph.D., Department of Oncology, University Hospital, SE-221 85, Lund, Sweden (e-mail: ake.borg@onk.lu.se).

See "Notes" following "References."

maining three families had been referred to the Oncogenetic Clinic, University Hospital, Lund, for genetic counseling. All families were selected on the basis of the occurrence of multiple cases of malignant melanoma and not for the presence of other malignancies. Mutation analysis was performed on a blood sample from a family member affected by malignant melanoma, typically the proband identified from the case–control study or the patient who was treated at the Department of Surgery or Dermatology. In families in which a CDKN2A mutation was found, additional members were identified and sampled for analysis, with the exception of a family that originated from Yugoslavia and could not be further extended. Two originally separate families with identical mutations were subsequently linked by parish records. The remaining families with mutation could not be connected to common ancestors. All diagnoses were confirmed by histopathologic analysis *(25).* All tested family members gave informed consent for the study, which was approved by the Ethical Committee of the Medical Faculty, Lund University.

All female family members (excluding spouses) identified and alive on January 1, 1958 (when the Swedish Cancer Registry was established), were followed through December 31, 1998, in the Swedish Census registry, the Swedish Cause of Death Registry, and the Swedish Cancer Registry with the use of a person-specific identification number.

Polymerase Chain Reaction–Single-Strand Conformation Polymorphism Analysis

DNA was extracted from blood with the use of the Wizard Genomic DNA Purification Kit (Promega Corp., Madison, WI) and from paraffin-embedded tumor and normal tissue as described previously *(26).* Polymerase chain reaction (PCR) primers flanking the coding regions and splice sites of exons 1–3 of the CDKN2A gene were used for amplification *(8).* The possible presence of a previously reported CDKN2A promoter mutation at position –34 was also checked by use of mutant and wild-type sequence-specific primers, as described elsewhere *(20).* Exon 2 was divided into three overlapping fragments so that no product exceeded 250 base pairs (bp). The PCR reactions were performed in 15 μL of 1× PCR buffer (i.e., 100 m*M* Tris–HCl [pH 8.3] and 500 m*M* Cl) containing 5% dimethyl sulfoxide (DMSO); 0.2 m*M* each of deoxyadensine triphosphate, deoxyguanosine triphosphate, and deoxythymidine triphosphate; 10 μ*M* deoxycytidine triphosphate (dCTP) (Amersham Pharmacia Biotech, Uppsala, Sweden); 0.09 μL [α-^{32}P]dCTP (10 μCi/μL, 3000 Ci/mmol; Amersham Life Science Inc., Arlington Heights, IL); 0.16 μ*M* of each primer; 1.5 m*M* $MgCl_2$ (The Perkin-Elmer Corp., Foster City, CA); 0.5 U AmpliTaq Gold (The Perkin-Elmer Corp.); and 100 ng genomic DNA. The PCR was carried out in a Touchdown (Hybaid, Ashford, Middlesex, U.K.) under the following conditions: enzyme activation at 93 °C for 11 minutes; 32–34 cycles of 93 °C for 30 seconds, 56 °C–63 °C for 30 seconds, and 72 °C for 50 seconds; and a final extension at 72 °C for 5 minutes. One microliter of PCR product was mixed with 9 μL denaturing loading buffer (95% formamide, 10 m*M* NaOH, 0.05% xylene cyanol, and 0.05% bromophenol blue), denatured at 95 °C for 5 minutes, and then cooled on ice. This mixture (0.5–1 μL) was loaded onto gels containing 0.5× MDE(tm) (mutation detection enhancement) gel solution (FMC Bioproducts, Rockland, ME), 0.6× Tris–Borate EDTA buffer, 0.08% ammonium persulfate, and 0.04% tetra methyl ethylenediamine. Single-strand conformation polymorphism (SSCP) analysis of the PCR products was performed both at room temperature (in gels containing 5% glycerol and run at 6 W for 14 hours) and at 4 °C (in gels containing 5% glycerol and run at 38 W for 4–6 hours), except for exon 1, for which the PCR products were better separated when the gel contained 10% glycerol and was run at 40 W for 9 hours. The first part of CDK4 (exon 2A), which encodes a region that interacts with $p16^{INK4a}$, was analyzed by PCR–SSCP by use of the same methods, except that no DMSO was added to the PCR *(24).*

Sequencing

All shifts found in PCR–SSCP analysis were further investigated by sequencing. A new PCR amplification of genomic DNA was performed as described above, except that one primer of each primer pair included an M13 sequence in the 5′ end. PCR products were used in the sequence reaction by use of the Dye Primer Cycle Sequencing Ready Reaction –21 M13 Kit (The Perkin-Elmer Corp.). The reactions were run on an Applied Biosystems 373 automated sequencer by use of premixed 6% sequencing gel solutions (Burst-Pak; Owl Separation Systems, Portsmouth, NH).

In Vitro Binding Assay

In vitro mutagenesis of CDKN2A was performed by use of the QuickChange Site-Directed Mutagenesis Kit (Stratagene Cloning Systems, La Jolla, CA) and oligonucleotides containing the desired mutation. *In vitro* binding assays of wild-type and mutant forms of $p16^{INK4a}$ to cdk2, cdk4, and cdk6 were performed as described elsewhere *(27).* Briefly, the following were subcloned into pBSKS(+): complementary DNAs (cDNAs) of wild-type CDKN2A; the site-directed mutants Arg87Pro, 113insArg, and Val115Gly; and CDK2, CDK4, and CDK6. The corresponding proteins were synthesized *in vitro* by use of the TNT expression system (Promega Corp.) with T7 polymerase in the presence of [^{35}S]methionine. Samples (2 μL) from the four $p16^{INK4a}$ variant reaction products were mixed individually with equal amounts of the cdk2, cdk4, and cdk6 proteins, and the mixtures were incubated for 30 minutes at 30 °C. The mixtures were then diluted to 1 mL with ice-cold buffer (500 m*M* NaCl, 1% [vol/vol] Nonidet P-40, and 3% [wt/vol] bovine serum albumin) and centrifuged at 1000*g* for 10 minutes at 4 °C to remove debris. Five microliters of polyclonal antiserum against $p16^{INK4a}$ (provided by G. Peters, Imperial Cancer Research Fund, London, U.K.) was added to the supernatant. The immune complexes were collected with protein A–Sepharose (Pharmacia Biotech, Inc.), analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis in 15% polyacrylamide gels, and visualized by autoradiography.

Statistical Analysis

Observed incidences for breast and pancreatic cancers among female family members were compared with expected incidences, calculated by use of age-, sex-, and calendar-year-specific reference data from the whole of Sweden. Tumors diagnosed before or after the study period were not included in the analysis. The risk of malignant melanoma was not analyzed because families were selected on the basis of family aggregation of this disease. Other malignant tumors were not included in the risk analysis because the numbers were too small. The risk calculations used the person-years method, in which individuals were classified into 5-year age groups and single calendar years were used as the unit cell size. Cause-specific standardized morbidity rates and 95% confidence intervals were calculated. *P* values were calculated by use of the chi-square distribution; $P<.05$ was considered to be significant. All tests were two-sided. Two-sided Fisher's exact test was used in the analysis of sex differences in propensity for multiple melanomas. Mann–Whitney's *U* test was used to compare the numbers of affected cases in CDKN2A mutation-positive and -negative families.

Results

Identification and Functional Analysis of CDKN2A Mutations

Fifty-two families were screened for mutations in the CDKN2A gene and part of the CDK4 gene by use of PCR–SSCP analysis. Cases manifesting an altered migration pattern during gel electrophoresis were further investigated by sequence analysis of a new PCR product from the same genomic DNA to determine the possible sequence variation. Two earlier described CDKN2A polymorphisms that do not affect function *(9),* an A→G transition in codon 148, leading to an exchange of alanine for threonine, and a C→G transition in the untranslated part of exon 3 at nucleotide position 494, were observed frequently. An earlier reported CDKN2A promoter variant at position −34 *(20)* was not found in any of the 52 families. The 5′ part of CDK4 exon 2, encoding a region that interacts with $p16^{INK4a}$, was also analyzed without finding any evidence of mutation *(24).*

Nine separate families, including two reported previously *(14),* were found to carry a 3-bp duplication that resulted in the insertion of an arginine at codon 113 (Fig. 1, A; Table 1). One additional family carried a different mutation—a T→G transition leading to the substitution of glycine for valine at codon 115. Both mutations reside in the fourth ankyrin repeat region, which is assumed to be involved in cdk4/cdk6 binding *(3).* To in-

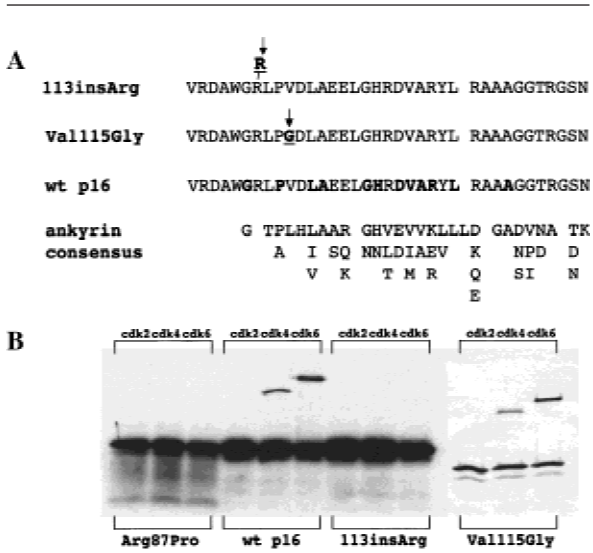

Fig. 1. A) Deduced sequence of the 113insArg $p16^{INK4a}$ mutant, the Val115Gly $p16^{INK4a}$ mutant, and the wild-type $p16^{INK4a}$ protein (wt p16) (residues 106–141, including the fourth ankyrin repeat, with conserved residues in **bold**). The **last three lines** show the consensus sequence of an ankyrin motif, including alternative amino acids at certain positions. **B)** Cdk binding properties of the control Arg87Pro mutant $p16^{INK4a}$ protein (which is known to be functionally impaired) *(27),* the wild-type $p16^{INK4a}$ protein, and the 113insArg and Val115Gly mutant $p16^{INK4a}$ proteins. Each variant was translated and radiolabeled *in vitro* and tested for the ability to bind to *in vitro* translated cdk2, cdk4, and cdk6 proteins. The products were immunoprecipitated with a polyclonal antiserum against $p16^{INK4a}$, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and visualized by autoradiography. The strongly labeled fast-migrating bands represent unbound $p16^{INK4a}$ protein. Wild-type $p16^{INK4a}$ protein and the Val115Gly variant retained the ability to bind cdk4 and cdk6, whereas Arg87Pro and 113insArg had no binding activity in this assay.

Table 1. Melanoma families with germline CDKN2A mutations

Family	Source*	CDKN2A mutation	Melanoma cases (males/females)	Multiple melanoma cases (males/females)	Pancreatic cancer cases	Breast cancer cases
Lund M2	A	113insArg	7 (3/4)	1 (0/1)	1	1
Lund M9	A	113insArg	2 (0/2)	1 (0/1)	2	1
Lund M11	B	113insArg	2 (2/0)	0	0	0
Lund M12	C	113insArg	3 (2/1)	0	1	1
Lund M13	C/D	113insArg	4 (1/3)	3 (1/2)	3	4
Lund M14	D	Val115Gly	2 (2/0)	0	0	0
Lund M31	D	113insArg	8 (4/4)	2 (1/1)	1	0
Lund M47	D	113insArg	5 (3/2)	0	1	0
Lund M49	D	113insArg	7 (2/5)	3 (0/3)	0	2
Lund M62	D	113insArg	3 (1/2)	1 (0/1)	0	1
Total			43 (20/23)	11 (2/9)	9	10

*Families were selected from one of four sources: A = patients treated at the Department of Surgery, Lund, Sweden; B = patients treated at a specialized melanoma clinic at the Department of Dermatology, Lund; C = patients referred to the Oncogenetic Clinic, Lund; and D = a population-based, case–control study of melanoma in southern Sweden.

vestigate whether the mutations affect the function of the encoded proteins, we used *in vitro* mutagenesis to introduce the base changes into the wild-type cDNA and a coupled transcription and translation system to express the proteins. The *in vitro* binding assay showed that the 113insArg mutant lacks the ability to bind cdk4 and cdk6 (Fig. 1, B), similar to a known functionally impaired mutant, Arg87Pro *(27).* The Val115Gly variant, by contrast, demonstrated no such loss of cdk4- or cdk6-binding capacity (Fig. 1, B). This mutant may disrupt some other aspect of $p16^{INK4a}$ function, but we cannot rule out the possibility that Val115Gly is just a polymorphism that has no effect on $p16^{INK4a}$ function.

Sex Difference in Propensity for Multiple Melanomas in CDKN2A-Positive Families

Families with identified CDKN2A mutations are drawn in Fig. 2 and are listed in Table 1. Of a total of 43 melanoma patients in CDKN2A mutation-positive families, 11—all in 113insArg families—were affected by multiple primary melanomas. The propensity for multiple melanomas in 113insArg families was different between the sexes: Nine of the 11 individuals with multiple melanomas were females compared with 14 of 30 individuals with a single melanoma (P = .075). Six of the nine 113insArg families included at least one case of multiple primary melanoma, and a total of 11 males and 20 females were affected by melanoma in these families. The ratio of affected males to affected females in these families was lower than that in the three 113insArg families without multiple melanomas, in which a total of seven males and three females were affected by melanoma (P = .075).

Occurrence of Breast and Pancreatic Carcinomas in CDKN2A-Positive Families

Six of the nine 113insArg families included one or more breast cancer cases, totaling 10 in all. It was possible to carry out mutation analysis on six of the 10 breast cancer patients, and all six were found to carry the 113insArg mutation (Fig. 2). Lund M13 included four breast cancer patients, one of whom was diagnosed at age 23 years and found to carry the 113insArg mutation. The age of onset for the remaining three breast cancer case patients in this family varied from 49 to 81 years; one of these patients also developed a phyllodes tumor at 48 years. Overall, among the six 113insArg families with breast cancer, three women developed both malignant melanoma and breast cancer. Moreover, breast cancer was observed in five of the six families with cases of multiple melanomas.

Pancreatic cancer was also common in 113insArg families. Six of the nine 113insArg families included a total of nine pancreatic cancer cases (three in males and six in females). Again, Lund M13 was extreme, with three cases. DNA was available from two of the nine pancreatic cancer patients (both from Lund M13), and both were found to be mutation carriers. Two of the nine patients with pancreatic cancer developed malignant melanoma. Other malignancies observed in the 113insArg families included carcinomas of the cervix, gallbladder, lung, larynx, prostate, ventricle, liver, and intestine, as well as cases of lymphoma and leukemia. None of these malignancies, however, occurred at the frequencies observed for breast and pancreatic carcinomas.

The observed versus expected cases of breast and pancreatic cancers were calculated for 80 females belonging to the nine families carrying the 113insArg mutation and eligible for the analysis. Of the 80 females (not all of whom are shown in Fig. 2), 22 were of known CDKN2A status (18 mutation carriers and four noncarriers) and 58 were of unknown CDKN2A

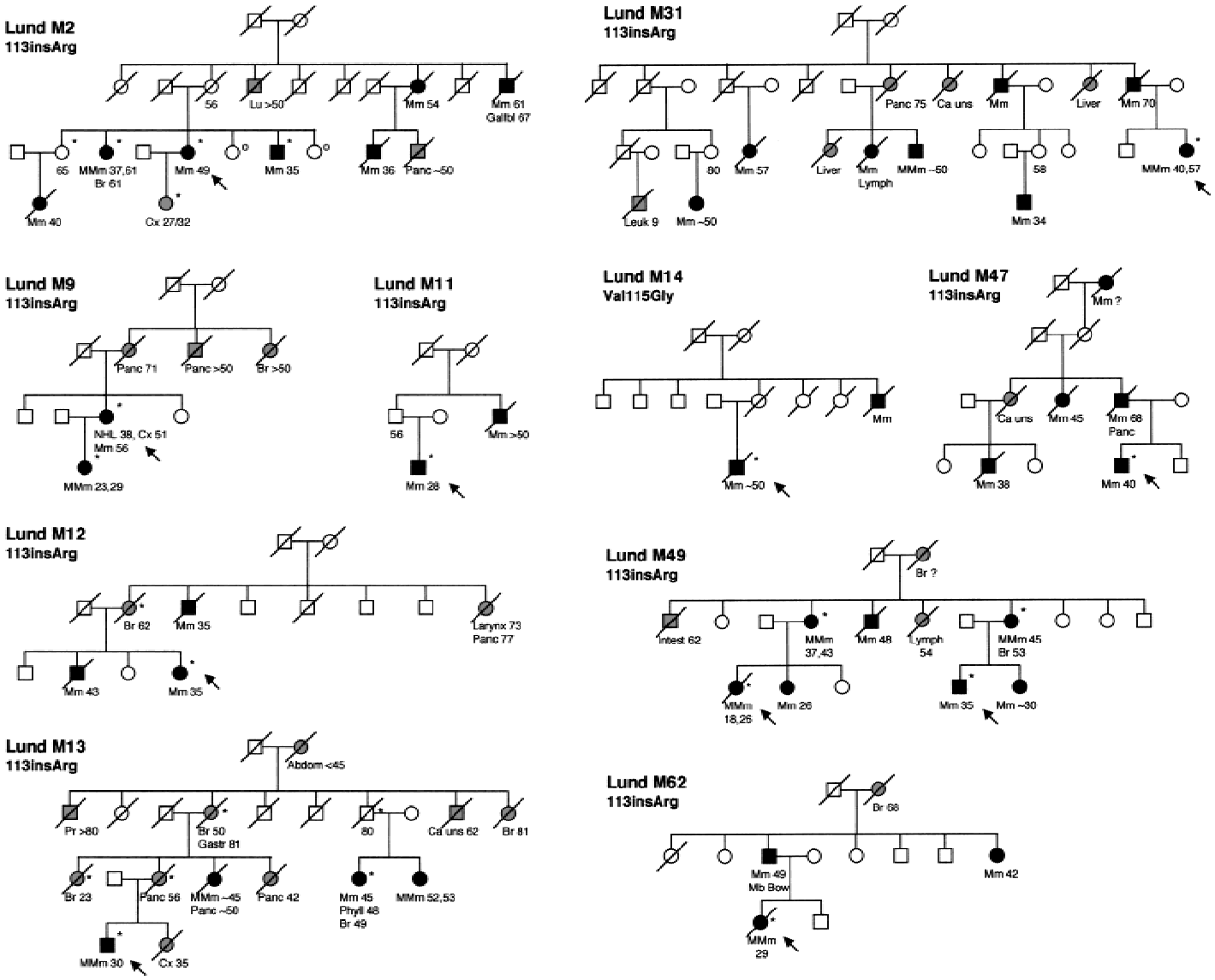

Fig. 2. Pedigrees of Swedish melanoma families with germline CDKN2A mutations. Lund M14 is of southern European origin and carries the Val115Gly variant. All of the other families carry the 113insArg mutation. Malignant diseases, age at diagnosis (or, for unaffected family member, present age), and mutation status (* = carrier, ◦] = noncarrier; remainder not tested) are indicated. Probands for the families and for mutation analysis are indicated by **arrows. Black symbols** indicate patients with malignant melanoma; **gray symbols** indicate patients with other kinds of cancer. Abdom = abdominal cancer of unknown type; Br = breast cancer; Ca uns = cancer of unknown type; Cx = cervical cancer; Gallbl = gallbladder cancer; Gastr = gastric cancer; Intest = intestinal cancer; Larynx = larynx cancer; Leuk = leukemia; Liver = liver cancer; Lu = lung cancer; Lymph = lymphoma; Mb Bow = Bowen's disease; Mm = malignant melanoma; MMm = multiple malignant melanoma; NHL = non-Hodgkin's lymphoma; Panc = pancreatic cancer; Phyll = phyllodes tumor; and Pr = prostate cancer.

Table 2. Observed risk of breast and pancreatic cancers in female members of melanoma families carrying the CDKN2A 113insArg mutation compared with expected numbers for Sweden*

	Observed	Expected	SMR	95% CI	P†
Overall					
Breast cancer	8	2.1	3.8	1.6–7.5	.0014
Pancreatic cancer	6	0.16	39	14–84	<.0001
Patients <55 y					
Breast cancer	4	1.1	3.8	1.0–9.6	.023
Pancreatic cancer	2	0.03	65	7.9–240	.0004

*SMR = standardized morbidity rate; CI = confidence interval.

†All P values are two-sided.

status. Eight breast cancer cases were observed compared with an expected number of 2.1, a statistically significant difference (P = .0014; Table 2). Similarly, the incidence of pancreatic cancer was significantly higher in these nine families than expected on the basis of the incidence in Sweden: six observed compared with an expected 0.16 (P<.0001). The increased risks of these malignancies were evident when studying all of the women as well as when restricting the analysis to younger women (<55 years of age; Table

2). The increased risks of breast and pancreatic cancers in women more than 55 years of age did not reach statistical significance (data not shown).

CDKN2A-Negative Families

In 42 of the 52 melanoma families, no CDKN2A mutation was found. Two previously characterized neutral polymorphisms (Ala148Thr and C→G at nucleotide position 494) were, however, repeatedly observed in these families. The number of melanoma cases in these families (mean, 2.3 cases; range, two through five cases) was significantly lower ($P = .001$) than in CDKN2A mutation-positive families (mean, 4.3 cases; range, two through seven cases). The numbers of breast and pancreatic cancer cases were also significantly lower ($P = .001$) in CDKN2A mutation-negative families (mean, 0.21 and 0.14, respectively) than in CDKN2A mutation-positive families (mean, 1.0 and 0.90, respectively). It should be emphasized that these calculations were not normalized for the numbers of members in each family, and the results should be interpreted cautiously.

Discussion

We screened 52 melanoma families for germline mutations in the CDKN2A and CDK4 genes by use of PCR–SSCP and sequence analysis. Ten (19%) of the 52 families were found to carry a CDKN2A mutation. None of the families had an alteration in CDK4 exon 2, which encodes the $p16^{INK4a}$-binding domain and has been found to be mutated in some cases of familial melanoma *(24).* In addition, none of the families contained the CDKN2A promoter mutation that has also been found in some cases of familial melanoma *(20).* Our screen did not include exon 1β of the CDKN2A locus, which encodes part of the $p14^{ARF}$ protein, or the CDKN2B locus. However, no germline mutation associated with familial melanoma has been observed in these regions *(7,13,19,28),* suggesting that the majority of families with 9p21-linked melanoma have mutations in CDKN2A exons 1α, 2, and 3, which were covered by this analysis.

Our study included 35 families identified from a population-based, case–control study of melanoma patients, representative of a southern Swedish cohort, as well as 17 families included by more selective criteria. These latter families represented consecutive cases treated for and reporting a family history of malignant melanoma (14 families) as well as individuals seeking genetic counseling because of a familial clustering of the disease (three families). In an earlier study of Swedish families with two or more first-degree relatives with melanoma *(15),* 8% (five of 64 families) had a CDKN2A mutation. In this study, 14% of the families identified in the case–control study (i.e., five of 35 families) were found to carry a CDKN2A mutation. Since this frequency represents data from a population-based series of families, it probably constitutes an accurate estimate of the proportion of melanoma families due to CDKN2A mutations. However, the majority of families with high numbers of members affected by melanoma (including five of the six families with four or more melanoma cases) were found to be mutation carriers, indicating that CDKN2A is the major melanoma susceptibility gene in high-risk families. Similar conclusions were reported recently from a study of Australian melanoma families *(29).*

Nine of the 10 families harboring a mutation in CDKN2A were found to carry a mutation, 113insArg, that has already been described in other Swedish families *(14,15).* The remaining family, which originated from southern Europe, included two affected cases and was found to carry a novel variant, Val115Gly. The 113insArg mutation has now been described in 17 Swedish families and represents a founder mutation of potential importance in the Scandinavian countries (Hashemi J, Bendahl PO, Sandberg T, Platz A, Linder S, Stierner U, et al.: unpublished data). A similarly strong founder effect for a CDKN2A mutation (19-bp deletion) has been reported previously from The Netherlands *(12).* Indeed, all 113insArg families have been shown to carry identical alleles for markers adjacent to the CDKN2A locus (Hashemi J, Bendahl PO, Sandberg T, Platz A, Linder S, Stierner U, et al.: unpublished data), which supports the hypothesis that the 113insArg mutation is a founder mutation.

The 113insArg mutation affects the fourth ankyrin repeat of the $p16^{INK4a}$ protein. Missense mutations in the ankyrin consensus sequence have been shown to affect the binding of $p16^{INK4a}$ to cdk4 *(7,27,30–36).* Our *in vitro* binding studies demonstrate that 113insArg has lost the ability to interact with cdk4 and cdk6, strongly suggesting that this mutant is disease causing. The Val115Gly mutant $p16^{INK4a}$, however, retains the ability to bind cdk4 and cdk6. Val115 is a nonconserved residue located in the fourth ankyrin repeat of the $p16^{INK4a}$ protein, and the Val-to-Gly substitution constitutes no dramatic shift in size or charge. Consequently, we cannot rule out the possibility that Val115Gly represents a benign polymorphism. Nevertheless, the mutant ought to be further investigated to clarify a possible role in melanoma susceptibility, since it might affect a $p16^{INK4a}$ function other than binding to cdk4 and cdk6. Inherited amino acid substitutions that alter the properties of the $p16^{INK4a}$ protein without preventing it from interacting with cdks have been described *(30).* Both 113insArg and Val115Gly reside downstream of the $p14^{ARF}$ open reading frame and affect the $p16^{INK4a}$ protein exclusively.

In agreement with previous reports *(18,37),* we saw a high prevalence of multiple melanomas in CDKN2A mutation-carrying families. It has been shown that 1%–5% of melanoma patients included in a database of 2600 consecutively registered melanoma cases in south Florida develop synchronous or metachronous primary melanoma *(38),* much less than the 26% (11 of 43 melanoma patients) observed in CDKN2A mutation-positive families in this study. Of interest, female carriers of the 113insArg mutation were more prone to develop multiple melanomas than men with the mutation. Earlier studies *(18,37)* have not reported a sex difference in the risk of developing multiple melanomas. Thus, melanoma development in patients with germline CDKN2A mutations may differ from that in patients with sporadic and non-CDKN2A hereditary melanoma. One possibility is that a sex-dependent factor (e.g., hormones) promotes or interacts with CDKN2A mutation-induced tumor progression.

An increased risk of pancreatic cancer in CDKN2A mutation carriers has been reported previously *(39–42).* Our results confirm this relationship and extend it to include breast carcinoma, which was the second most frequent malignancy (after melanoma) in 113insArg families and mutation carriers. An association between increased risks of breast cancer and of malignant melanoma has been suggested *(43).* However, an increased risk of breast

cancer in CDKN2A mutation carriers has not been reported in earlier studies of melanoma families, which may suggest that this association is mutation specific and due to perturbation of a $p16^{INK4a}$ activity other than inhibition of cdk4/cdk6. Nevertheless, our results reinforce the role of CDKN2A as a multiple tumor suppressor gene *(4)* and suggest that it should also be considered to be a breast cancer susceptibility gene. Moreover, breast cancer families with multiple cases of melanomas should be regarded as candidates for CDKN2A mutation screening.

REFERENCES

(1) Skolnick MH, Cannon-Albright LA, Kamb A. Genetic predisposition to melanoma. Eur J Cancer 1994;30A:1991–5.

(2) Cannon-Albright LA, Goldgar DE, Meyer LJ, Lewis CM, Anderson DE, Fountain JW, et al. Assignment of a locus for familial melanoma, MLM, to chromosome 9p13–p22. Science 1992;258:1148–52.

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

(4) Kamb A, Gruis NA, Weaver-Feldhaus J, Liu Q, Harshman K, Tavtigian SV, et al. A cell cycle regulator potentially involved in the genesis of many tumor types. Science 1994;264: 436–40.

(5) Nobori T, Miura K, Wu DJ, Lois A, Takabayashi K, Carson DA. Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. Nature 1994;368:753–6.

(6) Chin L, Merlino G, DePinho RA. Malignant melanoma: modern black plague and genetic black box. Genes Dev 1998;12:3467–81.

(7) Ruas M, Peters G. The p16INK4a/CDKN2A tumor suppressor and its relatives. Biochim Biophys Acta 1998;1378:F115–77.

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

(9) Kamb A, Shattuck-Eidens D, Eeles R, Liu Q, Gruis NA, Ding W, et al. Analysis of the p16 gene (CDKN2) as a candidate for the chromosome 9p melanoma susceptibility locus. Nat Genet 1994;8:23-6.

(10) Walker GJ, Hussussian CJ, Flores JF, Glendening JM, Haluska FG, Dracopoli NC, et al. Mutations of the CDKN2/p16INK4 gene in Australian melanoma kindreds. Hum Mol Genet 1995;4:1845–52.

(11) Holland EA, Beaton SC, Becker TM, Grulet OM, Peter BA, Rizos H, et al. Analysis of the p16 gene, CDKN2, in 17 Australian melanoma kindreds. Oncogene 1995;11:2289–94.

(12) Gruis NA, van der Velden PA, Sandkuijl LA, Prins DE, Weaver-Feldhaus J, Kamb A, et al. Homozygotes for CDKN2 (p16) germline mutation in Dutch familial melanoma kindreds. Nat Genet 1995;10:351–3.

(13) FitzGerald MG, Harkin DP, Silva-Arrieta S, MacDonald DJ, Lucchina LC, Unsal H, et al. Prevalence of germ-line mutations in p16, p19ARF, and CDK4 in familial melanoma: analysis of a clinic-based population. Proc Natl Acad Sci U S A 1996;93:8541–5.

(14) Borg A, Johannsson U, Johannsson O, Hakansson S, Westerdahl J, Masback A, et al. Novel germline p16 mutation in familial malignant melanoma in southern Sweden. Cancer Res 1996;56:2497–500.

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

(16) Harland M, Meloni R, Gruis N, Pinney E, Brookes S, Spurr NK, et al. Germline mutations of the CDKN2A gene in UK melanoma families. Hum Mol Genet 1997;6:2061–7.

(17) Soufir N, Avril MF, Chompret A, Demenais F, Bombled J, Spatz A, et al. Prevalence of p16 and CDK4 germline mutations in 48 melanoma-prone families in France. The French Familial Melanoma Study Group. Hum Mol Genet 1998;7:209–16.

(18) MacKie RM, Andrew N, Lanyon WG, Connor JM. CDKN2A germline mutations in U.K. patients with familial melanoma and multiple primary melanomas. J Invest Dermatol 1998;111: 269–72.

(19) Fargnoli MC, Chimenti S, Keller G, Soyer HP, Dal Pozzo V, Hofler H, et al. CDKN2a/p16ink4a mutations and lack of p19ARF involvement in familial melanoma kindreds. J Invest Dermatol 1998;111:1202–6.

(20) Liu L, Dilworth D, Gao L, Monzon J, Summers A, Lassam N, Hogg D. Mutation of the CDKN2A 5′ UTR creates an aberrant initiation codon and predisposes to melanoma. Nat Genet 1999;21:128–32.

(21) Bahuau M, Vidaud D, Jenkins RB, Bieche I, Kimmel DW, Assouline B, et al. Germ-line deletion involving the INK4 locus in familial proneness to melanoma and nervous system tumors. Cancer Res 1998;58:2298–303.

(22) Kumar R, Sauroja I, Punnonen K, Jansen C, Hemminki K. Selective deletion of exon 1 beta of the p19ARF gene in metastatic melanoma cell lines. Genes Chromosomes Cancer 1998; 23:273–7.

(23) Quelle DE, Zindy F, Ashmun RA, Sherr CJ. Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. Cell 1995;83:993–1000.

(24) Zuo L, Weger J, Yang Q, Goldstein AM, Tucker MA, Walker GJ, et al. Germline mutations in the p16INK4a binding domain of CDK4 in familial melanoma. Nat Genet 1996; 12:97–9.

(25) Mattsson B. Cancer registration in Sweden: studies on completeness and validity of incidence and mortality registers [doctoral thesis]. Stockholm (Sweden): Karolinska Institut; 1984.

(26) Lungu O, Wright TC Jr, Silverstein S. Typing of human papillomaviruses by polymerase chain reaction amplification with L1 consensus primers and RFLP analysis. Mol Cell Probes 1992;6:145–52.

(27) Parry D, Peters G. Temperature-sensitive mutants of p16CDKN2 associated with familial melanoma. Mol Cell Biol 1996;16: 3844–52.

(28) Liu L, Goldstein AM, Tucker MA, Brill H, Gruis NA, Hogg D, et al. Affected members of melanoma-prone families with linkage to 9p21 but lacking mutations in CDKN2A do not harbor mutations in the coding regions of either CDKN2B or p19ARF. Genes Chromosomes Cancer 1997;19:52–4.

(29) Aitken J, Welch J, Duffy D, Milligan A, Green A, Martin N, Hayward N. CDKN2A variants in a population-based sample of Queensland families with melanoma. J Natl Cancer Inst 1999;91:446–52.

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

(31) Liu L, Lassam NJ, Slingerland JM, Bailey D, Cole D, Jenkins R, Hogg D. Germline p16INK4A mutation and protein dysfunction in a family with inherited melanoma. Oncogene 1995;11:405–12.

(32) Sun S, Pollock PM, Liu L, Karimi S, Jothy S, Milner BJ, et al. CDKN2A mutation in a non-FAMMM kindred with cancers at multiple sites results in a functionally abnormal protein. Int J Cancer 1997;73:531–6.

(33) Ranade K, Hussussian CJ, Sikorski RS, Varmus HE, Goldstein AM, Tucker M, et al. Mutations associated with familial melanoma impair p16INK4 function. Nat Genet 1995;10: 114–6.

(34) Koh J, Enders GH, Dynlacht BD, Harlow E. Tumour-derived p16 alleles encoding proteins defective in cell-cycle inhibition. Nature 1995; 375:506–10.

(35) Lilischkis R, Sarcevic B, Kennedy C, Warlters A, Sutherland RL. Cancer-associated missense and deletion mutations impair p16INK4 CDK inhibitory activity. Int J Cancer 1996;66: 249–54.

(36) Yarbrough WG, Aprelikova O, Pei H, Olshan AF, Liu ET. Familial tumor syndrome associated with a germline nonfunctional p16INK4a allele. J Natl Cancer Inst 1996;88:1489–91.

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

(38) Brobeil A, Rapaport D, Wells K, Cruse CW, Glass F, Fenske N, et al. Multiple primary melanomas: implications from screening and follow-up programs for melanoma. Ann Surg Oncol 1997;4:19–23.

(39) Goldstein AM, Fraser MC, Struewing JP, Hussussian CJ, Ranade K, Zametkin DP, et al. Increased risk of pancreatic cancer in melanoma-prone kindreds with p16INK4 mutations. N Engl J Med 1995;333:970–4.

(40) Whelan AJ, Bartsch D, Goodfellow PJ. Brief report: a familial syndrome of pancreatic cancer and melanoma with a mutation in the CDKN2 tumor-suppressor gene. N Engl J Med 1995;333:975–7.

(41) Bergman W, Gruis N. Familial melanoma and pancreatic cancer. N Engl J Med 1996;334:471–2.
(42) Ciotti P, Strigini P, Bianchi-Scarra G. Familial melanoma and pancreatic cancer. Ligurian Skin Tumor Study Group. N Engl J Med 1996; 334:469–70; discussion 471–2.
(43) Hemminki K, Vaittinen P. Interaction of breast cancer and melanoma genotypes. Lancet 1997; 350:931–2.

NOTES

Supported by grants from the Swedish Cancer Society, the Nordic Cancer Union, the Mrs. Berta Kamprad Foundation, the Gunnar Arvid and Elisabeth Nilsson Foundation, the John and Augusta Persson Foundation, the Crafoord Foundation, the Hospital of Lund Foundations, the E. & F. Bergqvist Foundation, and King Gustav V:s Jubilee Foundation.

We thank Drs. Gordon Peters and Margarida Ruas, Imperial Cancer Research Fund, London, U.K., for providing reagents and technical support for the $p16^{INK4a}$ binding assay and Dr. David Hogg, King's College Circle, Toronto, ON, Canada, for primers and positive control DNA for the CDKN2A –34 promoter mutation.

Manuscript received September 9, 1999; revised May 17, 2000; accepted May 31, 2000.