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ARTICLE

Two p16 (CDKN2A) germline mutations in 30 Israeli melanoma families

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Germline mutations in the p16 (*CDKN2A*) tumour suppressor gene have been linked to inherited predisposition to malignant melanoma (MM). Variable frequencies of p16 germline mutations were reported in different collections of melanoma families but it can be as high as 50%. Here we describe the results of p16 mutation screening in 30 melanoma kindreds in Israel. The entire coding region of the p16 gene, including exons 1, 2 and 3, flanking exon/intron junctions, and a portion of the 3' untranslated (UTR) region of the gene were examined by single-stranded conformation polymorphism (SSCP) analysis and direct sequencing. Two p16 germline mutations were identified: G101W, which has been previously observed in a number of melanoma kindreds, and G122V, a novel missense mutation. Thus, the frequency of mutations identified in this collection of Israeli families was 7%. Functional analysis indicated that the novel G122V variant retained some capacity to interact with cyclin dependent kinases (CDKs) *in vitro*, yet it was significantly impaired in its ability to cause a G1 cell cycle arrest in human diploid fibroblasts. This partial loss of function is consistent with the predicted impact of G122V substitution on the 3-dimensional structure of the p16 protein. *European Journal of Human Genetics* (2000) **8**, 590–596.

Keywords: p16; CDKN2A; germline mutations; Israeli Jewish melanoma families

Introduction

The $p16^{INK4a}$-cyclin D1-CDK4-retinoblastoma protein (pRb) pathway is recognised as a major target of genetic alterations in diverse human cancers (see reviews[1,2]). One of the most frequently altered genes is the $p16^{INK4a}$/*CDKN2A* tumor suppressor which sustains inactivating lesions in a wide variety of sporadic tumours, notably T cell acute lymphoblastic leukemia (T-ALL), gliomas and bladder cancer, and is perturbed in 50–75% of established human tumour cell lines.[2,3] Germline mutations in p16 occur in families that show inherited predisposition to malignant melanoma.[2,4–6] and in some instances to pancreatic and head and neck cancers.[7,8]

About 230 melanoma families worldwide have been screened for germline alterations in p16 and 36 different mutations have been identified thus far.[2] The estimated frequency of p16 mutations in familial melanoma varies from 8 to 50%.[9–11] Some of this variability could reflect the different ways in which familial inheritance of melanoma is inferred. For example, in two studies a positive correlation was noted between the frequency of p16 mutation and the number of MM affected individuals within a given melanoma family.[10,11] Ethnic background may also be significant in determining the frequency of germline mutations, as was shown for several genes predisposing to cancer. For example, specific mutations in the *BRCA1* and *BRCA2* tumor suppressor genes causing breast and ovarian cancer have been

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found at elevated frequencies among Ashkenazi and non-Ashkenazi Jews.[12,13] Similarly, a hypermutable tract in the *APC* tumor suppressor gene occurs at a frequency of 28% in Ashkenazi Jewish individuals with family or personal histories of colorectal cancer.[14] With these precedents in mind, we have examined the prevalence and nature of p16 germline mutations in 30 Israeli melanoma families of both Ashkenazi and non-Ashkenazi Jewish origin. A preliminary account of these studies has been published elsewhere.[15]

Materials and methods

Melanoma families

Melanoma families were recruited from patient population consecutively followed at the Dermatology and Plastic Surgery Departments of three major Israeli Medical Centers: Sheba, Tel Hashomer, Sourasky, Tel Aviv and Hadassah Jerusalem. Ethnic origin was defined according to paternal and maternal grandparents. Ashkenazi Jews were classified as those who originate in Eastern and Central European countries. Non-Ashkenazi Jews were either of Sephardic (Spanish, North African, Balkan etc) or of Oriental (Iraqi, Iranian, Yemenite, Egyptian etc) origin. Kindreds examined in this study contained two, three, or four individuals with clinically and histologically confirmed MM. Two individuals with multiple primary melanomas were also examined. The presence of other cancers in the families studied was established through medical records and in part reported by patients themselves. DNA samples from only one MM affected member were available for mutation analysis in 25 families. In five families two or three MM affected individuals were examined for p16 germline alterations. All the participants enrolled in this study received a brief explanation of the aims of the study and signed an informed consent form. The study was approved by the internal review board/Helsinki committee of the participating Medical Centers.

Mutation detection

Genomic DNA was extracted from peripheral blood. PCR-SSCP analysis of the entire p16 coding region (including exon 1, exon 2 fragments 2A, 2B, 2C and exon 3) and an

Table 1 Summary of clinical data and p16 sequence alterations in melanoma probands

DNA sample	*Proband (age at diagnosis)*	*Ethnic origin*	*MM-affected relatives (age at diagnosis)*	*Other cancers in the family*	*Sequence alterations*[b]
AZ 01	Female (22)	Sephardic	Father (40), aunt (28)		442 G>A, Ala148Thr, 500C>G
M3	Male (35, 57)[a]	Oriental			
M6	Female (42)	Ashkenazi	Mother	Colon (mother), breast (aunt)	
M15	Female (46)	Ashkenazi	Father		
M29	Male (50)	Ashkenazi	Cousin		
M33	Male (44)	Ashkenazi	Mother, uncle		
M45	Male (22)	Ashkenazi	Mother		500 C>G
M46	Male (44)	Ashkenazi	Brother (46)	Brain (brother)	
M48	Male (80)	Ashkenazi	Sister (70)		
M50	Male (37)	Ashkenazi	Aunt, cousin (34)		
M51	Male (40)	Ashkenazi	Father, brother (39)		
M55	Female (39)	Sephardic	Twin sister (39)	Lymphoma (cousin)	
M57	Female (18)	Ashkenazi	Mother	Pancreatic (grandmother)	500 C>G
M59	Female (45)	Ashkenazi	Mother, cousin, nephew	Colon (mother), Pancreatic (sister), Breast (sister)	
M77	Male (43)	Sephardic	Mother		442 G>A, Ala48Thr, 500 C>G
1M	Female (43)	Ashkenazi	Sister (2 primaries)		
5M	Male (54)	Ashkenazi	Sister	Schwannoma (proband)	442 G>A, Ala148Thr, 500 C>G
21M	Female (55)	Italian	Mother		**301G>T, Gly101Trp**
22M	Female (31, 32)[a]	Sephardic & Oriental	Aunt (56)		
23M	Male (37)	Ashkenazi	Daughter (27)		442 G>A, Ala148Thr
24M	Female (46)	Ashkenazi	Daughter		
25M	Male (54)	Greek	Sister (41)		**365G>T, Gly122Val**
27M	Male (67)	Ashkenazi	Daughter (27)	Glioblastoma (brother)	
35M	Female (80)	Ashkenazi	Daughter (19)	Breast (daughter)	
37M	Female (35)	Ashkenazi	Mother		
40M	Female (44)	Ashkenazi	Father		
47M	Male (23)	Ashkenazi	Mother	Glioma (mother)	
48M	Male (60)	Ashkenazi	Brother (46)	Multiple basal cell carcinoma (brother)	
49M	Male (43, 57)[a]	Ashkenazi			
51M	Female (22)	Ashkenazi	Aunt, uncle		
52M	Female (48)	Oriental	Brother		
53M	Female (42)	Ashkenazi	Mother		442 G>A, Ala148Thr

[a]two primaries; [b]mutations are shown in bold.

untranslated 3' portion of the gene, direct sequencing of aberrant bands and restriction enzyme analysis were carried out as described by us earlier.[15] In addition, DNA from a subset of families of this collection was examined by direct sequencing of PCR products as described.[16] The –34G > T substitution in the 5' UTR of the p16 gene was assayed as previously described[16] except for changing the annealing temperature to 63 C.

Molecular modelling

The effect of G122V substitution on the three-dimensional structure of p16 protein molecule was examined by designing a model for p16 from Cα coordinates of p18 (kindly provided by Ronen Marmorstein). The model was drawn by using the RIBBON 2.5 program[17] and by omitting the fifth ankyrin repeat, which is not present in the p16 protein.[18]

Functional characterisation of the variant forms of p16

The functional evaluation of the G122V variant form was performed as described in detail elsewhere.[19] The G122V mutation was introduced into the wild-type p16 cDNA sequence using synthetic oligonucleotides containing the required base substitution and the QuickChange mutagenesis procedure according to the manufacturer's protocols (Stratagene, San Diego, CA, USA). Protein–protein interaction assays were performed using components synthesised *in vitro* by coupled transcription and translation of plasmid DNAs.[20] The CDKs were labelled by incorporation of ^{35}S-methionine. The protein complexes were immunoprecipitated with a polyclonal antiserum against the carboxy terminal region of p16, fractionated by SDS-PAGE in a 12% polyacrylamide gel and labelled components were visualised by autoradiography.

TIG-3 human diploid fibroblasts expressing the cell surface receptor for mouse ecotropic retrovirus (TIG-ER cells) were generated as previously described[21] and infected with recombinant retroviruses encoding wild-type and variant p16 sequences.[20] After 24 h, the medium was replaced and selection in medium containing 2.5 μg/ml puromycin, was initiated on day 2 post-infection and continued for a further 4 days. Equivalent numbers of puromycin resistant cells (5×10^3) were plated in 24-well plates and, at various times thereafter, cell numbers were compared by staining with crystal violet.[19] The relative numbers of viable cells were determined by measurements of absorbance at 590 nm. Values were normalised to that obtained at day zero and each time point was assayed in triplicate.

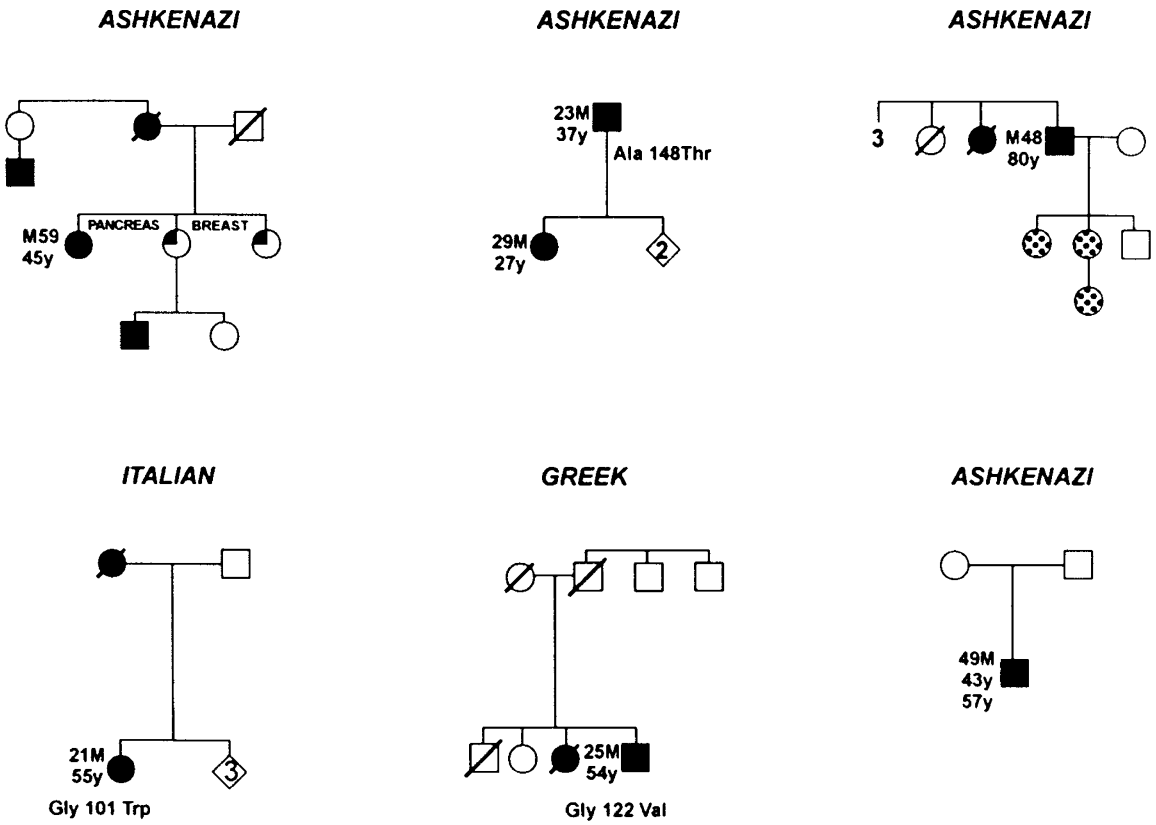

Figure 1 Six representative pedigrees of Israeli melanoma families. Filled squares and circles indicate males and females with MM. Upper quarter filled symbols indicate individuals with other cancers. Dotted symbols indicate presence of atypical mole syndrome. Ethnic origin, age at diagnosis (y) and sequence alterations are indicated.

Results

Melanoma-prone families

Thirty families were evaluated in the present study. One family contained four MM patients, five families, three patients and 24 families, two patients. In addition two patients with two primary melanomas were examined. In 27 families patients were first degree relatives, and in three families MM patients were second degree relatives. In nine families affected individuals were within the same generation, in 20 families within two generations, and one family MM spans three generations. Ages at diagnosis ranged from 18 to 80 years (mean = 41.4, 1SD = 14.6). From the data published by the Israel Cancer Registry on the newly diagnosed melanoma cases among Jews in 1988 we have estimated the average age at diagnosis as 54.4 years. Atypical Mole Syndrome (AMS) was assessed according to published criteria[22] in 15 families. AMS score equal to 3, conferring increased risk for melanoma was observed in three families. Other malignancies observed in related family members included: pancreatic cancer in two families, breast cancer in three families, brain tumours in four families, colon cancer in two families and other cancers in three families. Twenty-four families were of Ashkenazi-Jewish origin and the remaining eight families were of non-Ashkenazi origin. The relevant clinical details on the Israeli melanoma prone families are presented in Table 1 and pedigrees of six representative families are depicted in Figure 1.

p16 nucleotide sequence alterations

DNA from 37 MM patients representing 30 families and two individuals with multiple primary melanomas was examined by SSCP analysis followed by nucleotide sequence analysis. Sequence variations were identified in nine families (Table 1). Direct sequencing of all exons and exon–intron boundaries of the p16 gene revealed no additional sequence variations in a subset of 15 families.

A novel missense mutation, 365 G > T, coding for Gly122Val amino acid substitution was identified in a Sephardic-Jewish family (25M) originating from Salonika, Greece (Figure 1 and Figure 2). The proband, a 54-year-old male, was diagnosed having MM and AMS score 2. His sister

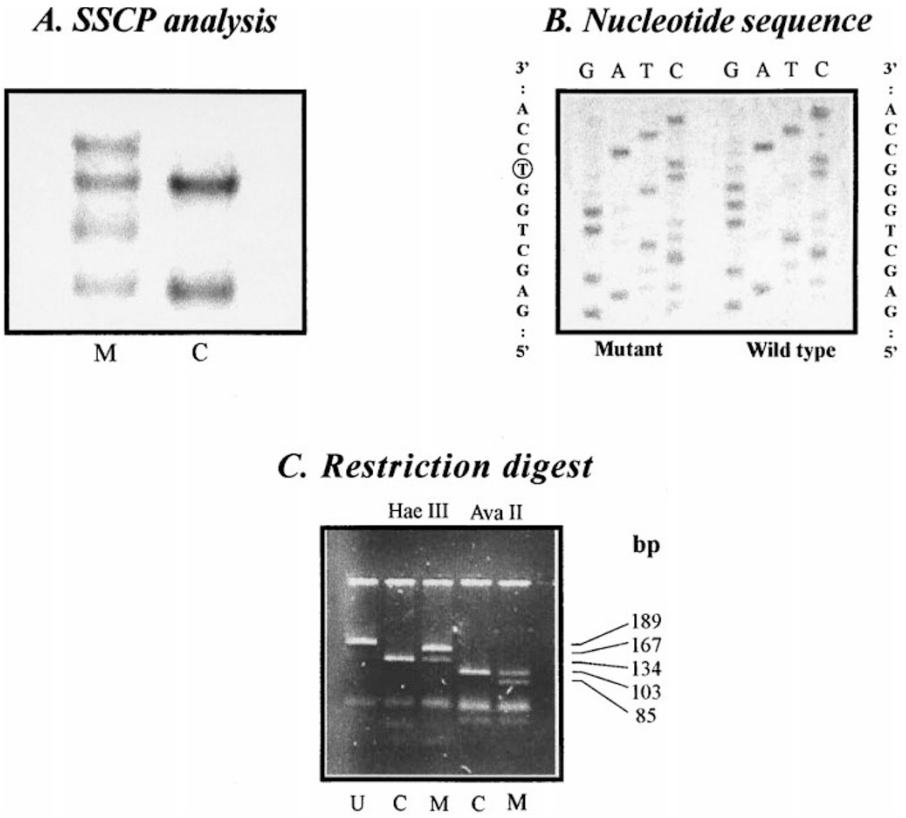

Figure 2 Identification of the 365G > T (Gly122Val) mutation **A** SSCP pattern of PCR fragment 2C of exon 2. M, individual 25M affected with melanoma (see pedigree in Figure 1); C, control **B** Nucleotide sequence of aberrant (M) and control (C) SSCP alleles. The G to T change is indicated by a circle **C** Confirmation of nucleotide sequence alteration by restriction analysis with HaeIII (GG*CC) and AvaII (G*GTCC) in control (C) and patient 25M (M) genomic DNA. The 189 bp long, undigested PCR fragment 2C is shown in lane U. Digestion by Hae III results in fragments of 134, 33, and 22 bp for wild type, and fragments of 167 and 22 bp for mutated alleles respectively. Digestion by Ava II results in fragments of 103, 51 and 35 bp for wild type allele and fragments of 85, 51, 35 and 18 bp for mutated allele.

died from MM at the age of 45 having been diagnosed at the age of 41.

A second missense mutation, 301 G > T, encoding for Gly101Trp amino acid substitution was identified in patient 21M of Italian Jewish origin (Figure 1). The proband was a 55-year-old female (AMS score 0), whose mother died from MM. The Gly101Trp mutation is a known melanoma causing mutation, which was detected in several families.[3,4,7,11,23]

The Ala148Thr polymorphism was identified in five out of the 32 probands (15.6%, Table 1). The frequency of this polymorphism in a control population sample was 8% (8/100). In two families, AZ01[15] and 23M (Figure 1) this polymorphism did not co-segregate with the disease. The 3'UTR 500 C > G polymorphism was identified in five instances (Table 1). The recently identified –34 G > T mutation in the 5' UTR of the p16 gene[16] was not detected in all the patients analysed.

The effect of the Gly122Val mutation on the structure and function of the p16 protein

The p16 protein belongs to a group of ankyrin motif containing proteins.[24] Four ankyrin repeats comprise almost 80% of the p16 molecule length. The availability of the three dimensional structure of the related human p18 protein allowed us to examine the possible effect of the Gly122Val mutation on the p16 molecule.[18] Glycine at position 122 facilitates the formation of a sharp turn between the α7 and α8 antiparallel helices in the fourth ankyrin repeat of the p16 protein.[18] Its replacement by valine, which exposes a hydrophobic side chain towards the solvent, will apparently lead to the destabilisation of the ankyrin molecule (Figure 3).

The functional evaluation of the Gly122Val variant protein was carried out by testing its ability to bind to CDK4 and CDK6, and its ability to cause a G1 cell cycle arrest in a human diploid fibroblast proliferation assay. Whilst binding of the variant protein to CDK6 was similar to that of the wild type protein, binding to CDK4 was significantly reduced (Figure 4A). The mutant protein was also less effective than the wild type protein in its ability to inhibit fibroblast proliferation (Figure 4B).

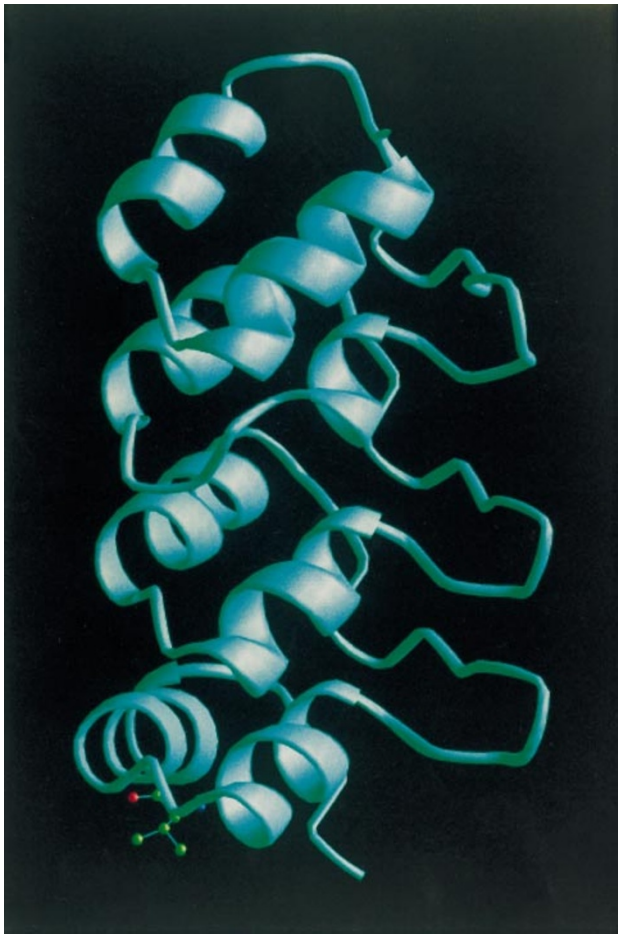

Figure 3 The effect of Gly122Val amino acid substitution on the 3-dimensional structure of p16 protein molecule. A ribbon model of p16 was derived from Cα coordinates of p18 omitting the fifth ankyrin repeat, which is not present in p16. The 122Val residue is illustrated by ball-and-sticks. It can be seen that the Val residue is surface located at the turn between the 7th and 8th α-helices. This location and solvent accessibility of the hydrophobic side chain of valine apparently destabilises the ankryin motif.

Discussion

Two p16 germline mutations were detected in 30 Israeli Jewish melanoma-prone families. A novel G122V missense mutation was identified in a 54-year-old patient whose sister died of melanoma at the age of 45 (family 25M in Figure 1). Several lines of evidence suggest that the G122V alteration is not a polymorphism but rather a disease-causing mutation. This mutation was not detected in any other MM patient studied, and was not detected among 30 unrelated sporadic melanoma patients or in 56 control healthy Jewish individuals. Glycine 122 is within the consensus ankyrin motif sequence. It is fully conserved in the INK4 proteins p15, p16, p18 and p19[18] and is located at the turn between two antiparallel α-helices. The G122V substitution apparently causes destabilisation of the p16 protein because of the relatively bulky side chain of valine as compared with that of glycine (Figure 3). Moreover, in functional tests, the G122V mutation impairs the binding of the p16 protein to CDK4 and its ability to cause a G1 cell cycle arrest in human diploid fibroblasts. A similar glycine to serine substitution (G122S) was reported as a somatic mutation in a biliary tract tumor.[25]

The G101W mutation has been previously described in a number of melanoma kindreds,[3,4,7,11,23] including seven unrelated families in Italy.[23] Since the family 21M, harbouring this mutation is of Italian origin, further studies comparing p16 gene linked haplotypes in the Italian and Israeli MM

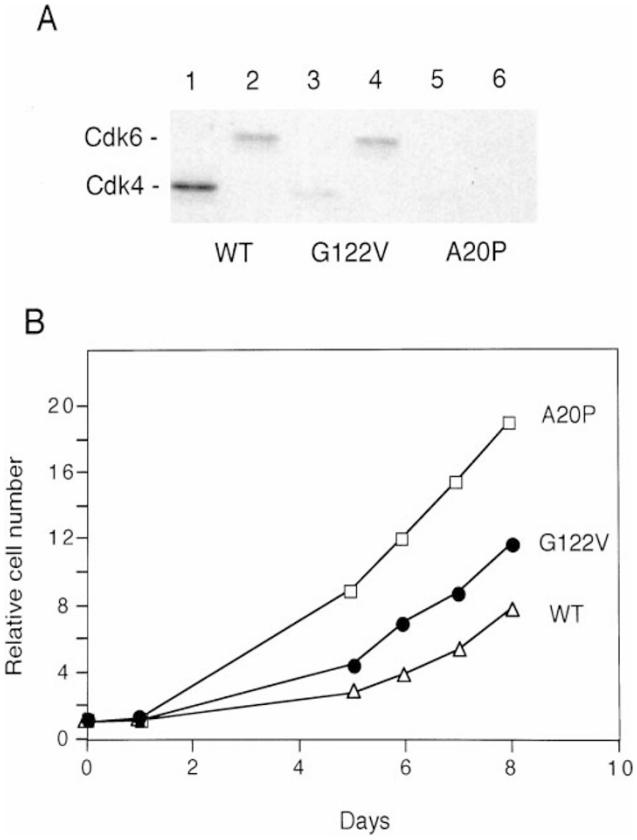

Figure 4 Functional evaluation of p16 variants **A** The ability of p16 variants to bind to CDK4 (lanes 1, 3 and 5) and CDK6 (lanes 2, 4 and 6) was tested by mixing unlabeled p16 with ^{35}S-labelled CDKs, produced by *in vitro* translation in reticulocyte lysates. The mixtures were immunoprecipitated with antiserum against p16 and the co-precipitated CDKs were analysed by SDS-PAGE and visualised by autoradiography. WT refers to wild-type p16 and A20P is a known loss of function mutant (19) **B** Inhibition of proliferation of TIG-3 human diploid fibroblasts infected with recombinant retroviruses encoding wild-type and variant p16 sequences. Cell proliferation assays were performed as described in Materials and methods.

affected individuals may clarify whether there is a common origin (founder effect) of this mutation.

Familial predisposition to melanoma is often but not always associated with the Atypical Mole Syndrome (AMS).[22,26,27] In our study, both the G122V and the G101W mutation carriers did not exhibit significant AMS scores (AMS 2 and AMS 0, respectively).

The frequency of p16 germline mutations identified in Israeli melanoma families appears to be low (2/30, 7%). In similar collections of Swedish, UK and French melanoma families, the frequency of *p16* gene mutations ranged from 8 to 50%.[9–11] The low frequency of detected mutations in the present study is apparently not a result of missed point mutations or small gene aberrations since the results of full DNA sequencing in 15 patients was concordant with the results of SSCP analysis. We cannot exclude, however, that some of the tested individuals represent sporadic melanoma cases especially in the families where only one individual was available for testing.

In previous reports[10,11] the majority of mutations were detected in families with three or more cases of melanoma. In our series, which included six families with three or more melanoma cases, p16 germline mutations were identified only in families with two melanoma cases. The majority of the families (23/30) in this collection were of Ashkenazi Jewish origin. In contrast with the high frequency of *BRCA1* and *BRCA2*, founder mutations causing breast and ovarian cancer in Ashkenazi Jews[28] no such mutations have been found yet in the p16 gene. In other populations, founder mutations in the p16 gene were reported on several occasions.[16,23,29–32]

Examination of a greater number of melanoma families, extension of analysis to non-coding regions of the p16 gene and screening for large rearrangements involving the p16 gene, will further clarify the role of p16 mutations in familial melanoma in Jews. The possibility of presence of germline mutations in another gene, *CDK4*, which have been reported in a few melanoma pedigrees[11,33] should be also investigated.

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