Analysis of the p16 gene (*CDKN2*) as a candidate for the chromosome 9p melanoma susceptibility locus

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A locus for familial melanoma, *MLM*, has been mapped within the same interval on chromosome 9p21 as the gene for a putative cell cycle regulator, p16^{INK4} (*CDKN2*) MTS1. This gene is homozygously deleted from many tumour cell lines including melanomas, suggesting that *CDKN2* is a good candidate for *MLM*. We have analysed *CDKN2* coding sequences in pedigrees segregating 9p melanoma susceptibility and 38 other melanoma-prone families. In only two families were potential predisposing mutations identified. No evidence was found for heterozygous deletions of *CDKN2* in the germline of melanoma-prone individuals. The low frequency of potential predisposing mutations detected suggests that either the majority of mutations fall outside the *CDKN2* coding sequence or that *CDKN2* is not *MLM*.

Susceptibility to melanoma has a significant genetic component, apart from skin coloration and melanin composition. A locus responsible for melanoma predisposition, *MLM*, has been mapped to a 2 centiMorgan (cM) interval in chromosome 9p21 between *D9S171* and *D9S736* (refs 1–5). This locus is believed to act as a somatically recessive tumour suppressor gene in the manner originally proposed by Knudson[6]. Inheritance of a single defective *MLM* allele predisposes to melanoma, while somatic loss or mutation of the second allele completes one of the steps necessary for development of malignancy. Based on this two-step model for carcinogenesis, somatic mutations are predicted to occur in neoplastic cells at the location of *MLM*.

Consistent with this view, chromosome 9p21 is the site of frequent somatic chromosomal aberrations in tumour cells and cell lines. Nearly 60% of melanoma tumour cell lines have homozygous deletions in 9p21, a finding that strongly suggests the presence of a tumour suppressor gene in 9p21 (ref. 7). Recently, a gene named *CDKN2* was localized within the deleted region[8–9]. *CDKN2* encodes a protein, p16, previously identified by its ability to bind and inhibit cyclin-dependent kinases (CDKs) *in vitro*[10]. CDKs, along with their associated positive regulatory factors, the cyclins, are principal determinants of the decision to initiate DNA replication and mitosis (for review, see ref. 11). As it encodes a putative inhibitor of cell division, *CDKN2* has the hallmarks of a tumour suppressor gene.

CDKN2 consists of three coding exons: exon 1 (E1) containing 125 bp, exon 2 (E2) 307 bp and exon 3 (E3) just 12 bp[8] *CDKN2* is deleted homozygously in a wide variety of tumour cell lines, including melanomas[8,9]. In addition, a high percentage of melanoma lines that do not contain homozygous deletions contain smaller hemizygous genetic lesions in either E1 or E2 of *CDKN2* such as frameshift, nonsense and missense mutations[8]. Similar mutations are seen in primary and metastatic melanomas (N.A.G. *et al.*, manuscript submitted). These results suggest that *CDKN2* is involved in formation of melanomas and, as such, is an appealing candidate for *MLM*. To test whether or not *CDKN2* is *MLM*, we have examined *CDKN2* for mutations that segregate with predisposition in melanoma-prone families.

Analysis of *CDKN2* in 9p21-linked families

Individuals belonging to melanoma-prone families that show evidence of 9p21-linked susceptibility were screened for predisposing mutations in *CDKN2* (Table 1). Eight of these families were American (7 from Utah, 1 from Texas[12]), exhibiting a combined multipoint lod score of +12.8 for localization between the 9p21 markers *IFNA* and *D9S126* (ref. 1). Five Dutch families with a combined lod score of +3.52 for *D9S171* were also examined[3]. Although in some kindreds the lod scores are low, or slightly negative, each kindred showed significant haplotype sharing among melanoma cases for the *IFNA–D9S126* region (Table 1), and were thus considered putative 9p-linked kindreds.

For all samples the same screening strategy was used: The entire coding sequence of *CDKN2* including adjacent splice junctions was amplified in three parts corresponding to E1, E2 and E3, using oligonucleotide primers flanking the exons. DNA sequence analysis from the various samples revealed three heterozygous nucleotide substitutions in E2 among the eight American probands. (No *CDKN2*

Table 1 Evidence for 9p linkage in putative 9p-linked kindreds

Nationality	Kindred	Lod score	Cases total (n)	Cases with haplotype (n)
American	3346	5.97	21	21
	1771	3.57	12	12
	3137	1.90	17	16
	1764	1.04	4	4
	3012	0.64	4	4
	3006	0.19	6	3
	3343	–0.53	10	8
	3161	–0.01	10	8
Dutch	4	1.22	6	6
	10	0.97	5	5
	1	0.95	4	4
	6	0.62	4	4
	3	–0.24	5	4

The lod scores were calculated for marker *D9S171* in Dutch families, and between markers *IFNA* and *D9S126* in American families.

sequence differences were detected in the Dutch individuals.) The three mutations detected in the American individuals were single nucleotide, missense mutations. One, in kindred 3012 (Gly93Trp), resulted in the substitution of a small, neutral amino acid by a large hydrophobic residue. Another, in kindred 1771 (Val118Asp), involved the replacement of a small hydrophobic residue by an acidic residue. The third variant, in kindred 3343 (Ala140Thr), involved a more conservative substitution.

Thirty-eight additional affected individuals (28 from Utah, ten from Australia) with a positive family history for melanoma from kindreds that had not been tested for 9p linkage were also screened for *CDKN2* mutations. The 28 Utah kindreds consisted of 26 with an average of more than three melanoma cases per kindred (range 2–10 cases), and two kindreds with one melanoma case and two or more cases of dysplastic nevus syndrome, a condition characterized by frequent occurrence of abnormal skin moles[3]. The ten Australian kindreds were ascertained for a significant excess of melanoma cases and have an average of more than three melanoma cases per kindred (range 3 to 4 cases)[13]. E1 and E2 were amplified from the genomic DNA of these individuals and subjected to DNA sequence analysis. No further polymorphisms were observed.

Population frequency of *CDKN2* substitutions

To test whether or not the missense substitutions observed in the melanoma-prone individuals were common polymorphisms, a population frequency analysis was conducted in unrelated individuals who had married into high risk cancer kindreds studied in Utah, but who themselves had no apparent increased risk of cancer. Genomic DNA from this normal set was used to amplify the E2 fragment from *CDKN2*. These fragments were probed with allele-specific oligonucleotides (ASOs) designed to detect each of the three missense changes. Two of the variants (Gly93Trp, Val118Asp) were not detected in the set of 100 normal samples, while the third (Ala140Thr) was present in 6/163 of the samples. These results suggest that the Ala140Thr missense change is a moderately common polymorphism present in roughly 4% of the Utah population and is unlikely to be a predisposing mutation. The other two missense mutations were rare in the normal population and were, therefore, candidates for predisposing mutations.

To determine whether the Gly93Trp and Val118Asp substitutions were present in other familial melanoma cases and in sporadic melanoma cases, we performed further ASO experiments. Thirty affected individuals (for Val118Asp) and 51 (for Gly93Trp) with positive family histories for melanoma from Australian families in which linkage analysis had not been performed were screened for these mutations. Also, 66 affected individuals (for Va118Asp) and 20 (for Gly93Trp) with unknown family history from Utah and Australia were analysed. No other occurrences of the mutations were detected, suggesting that they are rare in familial and sporadic melanoma cases.

Segregation analysis of *CDKN2* mutations

If the Gly93Trp and Val118Asp mutations are predisposing, they should segregate with melanoma susceptibility in the respective kindreds. To test linkage to the chromosome carrying the melanoma predisposition in kindreds 3012 and 1771, genomic DNA from available related individuals in each kindred was used to amplify E2 sequences for DNA sequence or ASO analysis. In both kindreds, the mutations were present in the *MLM* carrier individuals and not in noncarriers, demonstrating cosegregation of *MLM* with the two mutations (Fig. 1). This finding is consistent with the possibility that these two missense mutations Gly93Trp in family 3012 and Val118Asp in family 1771 may be predisposing *MLM* mutations.

Analysis of *CDKN2* germline deletions

Large germline deletions are the exception rather than the rule in familial cancers. Nevertheless, the prevalence in tumour cell lines of *CDKN2* homozygous deletions, some of which stretch several megabases, suggested that hereditary predisposition to melanoma might involve germline deletions in melanoma-prone families[7,14]. To test this possibility, probands and relatives from seven American 9p21-linked families and 21 unrelated individuals from 18 Utah melanoma kindreds were examined. Southern blots were prepared using genomic DNA digested with *Bam*HI or *Pvu*II. The blots were probed with cosmid c5, a cosmid that includes E1 and E2 of *CDKN2* and E2 from a related gene, *MTS2*. The blots revealed the presence of restriction fragment length polymorphisms (RFLPs) that could distinguish one homologue from the other in this region (Fig. 2 shows *Bam*HI digests). These RFLP patterns were interpreted as allele types and their frequencies determined.

If a significant fraction of the samples contained large heterozygous deletions of this region, the distribution of homozygotes compared to heterozygotes should deviate from the distribution predicted by the Hardy-Weinberg law. The observed gene frequencies for the rarer allele were 0.40 for the *Bam*HI polymorphism and 0.18 for the *Pvu*II polymorphism. In each case, the genotype frequencies fit the Hardy-Weinberg equilibrium (χ^2 p = 0.98 and p = 0.60, respectively). This suggests that the region does not contain large deletions in a significant fraction of melanoma-prone kindreds. Moreover, no

RFLPs other than the two allelic types were detected. This excludes the possibility of smaller deletions on the order of tens to thousands of base pairs which would have generated novel restriction fragments within the set of fragments detected by cosmid c5.

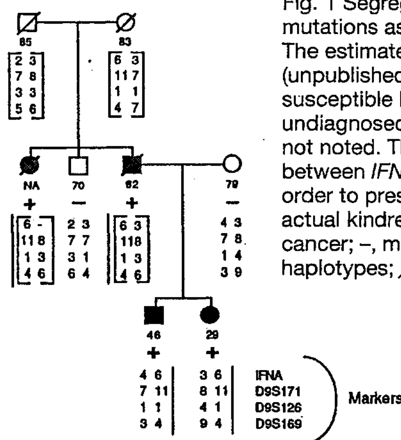
Discussion

So far, ten familial cancer genes have been cloned and characterized[15]. In each case where the gene has been analysed extensively, it is involved in sporadic cancer, as well as in hereditary cancer. For example, mutant forms of *p53* cause a proportion of the rare familial Li-Fraumeni cancer syndrome, while *p53* is mutated in nearly 50% of sporadic human cancers[16] Similarly, a gene that contributes to hereditary melanoma such as *MLM* is expected to be mutated in some sporadic cancers as well. *MLM* maps to a region that may encompass over a megabase in chromosomal region 9p21. *CDKN2*, deleted homozygously or mutated in nearly 75% of melanoma cell lines, maps within the same interval and is, therefore, an ideal candidate for *MLM*.

In a search for predisposing mutations in 9p-linked melanoma-prone families, we have found two potential *CDKN2* mutations. Both were linked to the carrier chromosome and neither was detected in the normal population. Neither involved a conservative amino acid substitution. Thus, these two mutations satisfy several important criteria for predisposing mutations. In addition, a previous study reported a nonsense mutation in a lymphoblastoid cell line derived from an individual with dysplastic nevus syndrome[9]. However, this finding is based on the analysis of DNA from cultured cells and it is unknown whether or not the mutation was present in the germline of the individual. In our screen of *MLM*-linked pedigrees, no unquestionably disruptive *CDKN2* mutations such as nonsense or frameshift mutations were found. This finding contrasts with the observation of point mutations in melanoma cell lines where 11/18 changes from the wildtype sequence caused premature termination of the p16 protein[8].

Could the two missense mutations detected in kindreds 1771 and 3012 be neutral? The p16 protein encoded by *CDKN2* consists of four tandemly repeated ankyrin motifs that together account for 88% of the total sequence[10]. Both the putative Gly93Trp and Val118Asp mutations disrupt the ankyrin repeat consensus. In contrast, the Ala140Thr substitution (also found in healthy individuals) lies outside the ankyrin domains. This is consistent with the rare mutations affecting p16 function, and the common polymorphism having no effect. On the other hand, the p16 protein is only 148 residues long, which implies a large surface to volume ratio, increasing the probability that an amino-acid change would occur on the exterior of the protein, and hence, be less disruptive to the folded state of the molecule. Therefore, in the absence of biochemical or structural information, the possibility that the two rare missense mutations are neutral cannot be excluded.

Kindred 3012



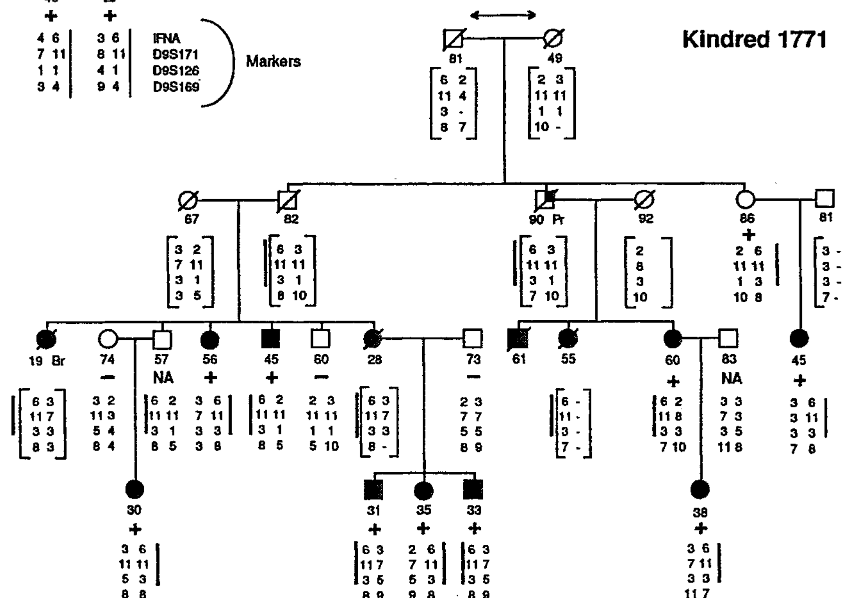


Fig. 1 Segregation of the susceptible 9p21 haplotype of four DNA markers and the *CDKN2* mutations as determined by DNA sequence or ASO analysis in kindreds 3012 and 1771. The estimated melanoma penetrance of MLM in the set of Utah kindreds is 53% by age 80 (unpublished data) resulting in the observation of some unaffected carriers of the susceptible haplotyped and mutation who are assumed to be either non-penetrant or undiagnosed individuals. Other cancers are shown: Pr, prostate. Recombinant events are not noted. The solid vertical bar identifies the segregating 9p21 haplotype; *MLM* maps between *IFNA* and *D9S171*. The gender or age onset has been changed in some cases in order to preserve confidentiality (age onset reflects the accurate decade in all cases). The actual kindred data is available upon request. ●, Melanoma; 24 Pr, age diagnosis, other cancer; –, mutation screened negative; +, mutation screened positive; [ ], inferred haplotypes; ⍁, deceased.

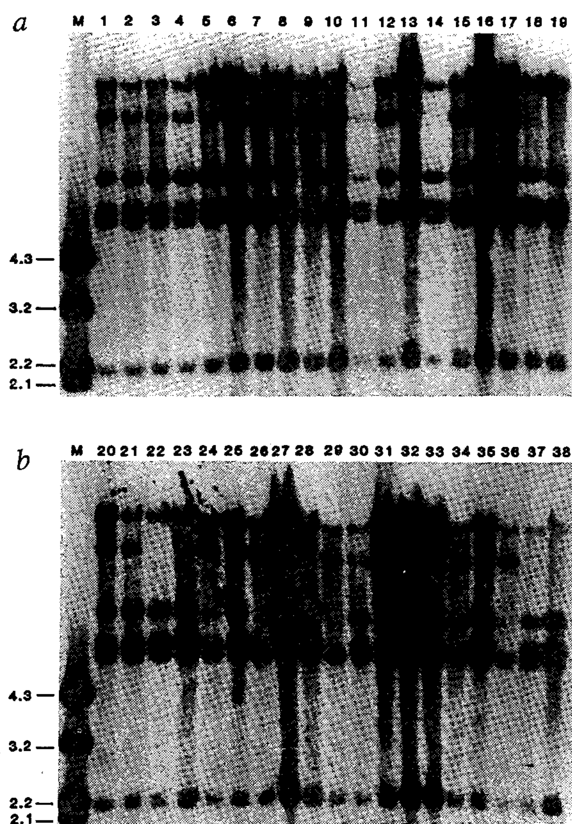


Fig. 2 Autoradiograph of Southern blot probed with cosmid c5. *a*, Lanes 2 and 6 are control DNA samples from noncarriers. *b*, Lanes 3 and 4 are control DNA samples from noncarriers. 5 µg genomic DNA was digested with *Bam*HI and loaded onto a 0.7% agarose gel. Lanes other than the control lanes contain DNA from either 9p-linked, predisposition carriers or from affected individuals from melanoma-prone families. Genotypes were assigned as Aa for lane 1, aa for lane 5, and AA for lane 9, for example; A is the rarer allele.

Regardless of whether or not these changes are neutral, the frequency of *CDKN2* mutations observed in melanoma-prone kindreds was much lower than would be expected if *CDKN2* were *MLM*: 2/13 in 9p21-linked families and 0/38 in familial melanoma cases while it is possible that not all 13 kindreds are linked, several of these kindreds have been shown previously to segregate a locus for melanoma predisposition at 9p21 with a high degree of statistical confidence. For example, kindreds 3346 and 3137 have lod scores in favour of 9p21 linkage of +5.97 and +1.90, respectively[1]. Evidence for genetic heterogeneity in melanoma predisposition involving a 1p locus has been presented[4,17,18]. However, there is no evidence in Utah, Dutch and Australian families for 1p-linkage or genetic heterogeneity in melanoma predisposition[1,19–23]. Even allowing for sporadic melanoma incidence and genetic heterogeneity, the frequency of germline *CDKN2* mutations is low.

There are two possible explanations for the low frequency of *CDKN2* mutations observed: either *CDKN2* and *MLM* are distinct genes or *CDKN2* is *MLM*, but the majority of predisposing mutations occur outside the p16 coding region and adjoining splice junction sequences. The authors of two recent reports argue that a second tumour suppressor gene may lie in 9p21 based on analysis of several non-melanoma primary tumours and that homozygous deletions of *CDKN2* in tumour cell lines may be partly an artifact of growth in culture[24,25]. However, other studies do not support this possibility[26,27]. Because of its *a priori* strength as a candidate, it is difficult to exclude *CDKN2* categorically and conclude that *MLM* is a different gene in 9p21. The precedent is compelling that in transformed cells somatic lesions occur in tumour suppressor genes, some alleles of which predispose to cancer. In the case of *CDKN2*, the homozygous deletion frequencies of individual members of a set of markers from 9p21 peak within the *CDKN2* gene[8]. The only other candidate gene identified so far in the immediate vicinity of *CDKN2* is its relative, *MTS2*. E2 sequences of this gene have been exhaustively screened for mutations in both pedigrees and melanoma lines[8]. The only polymorphisms identified so far were common and fell in a presumptive intron well outside the equivalent of E2 from *MTS2*. Thus, based on homozygous deletions in tumour cell lines and DNA sequence analysis, there is no evidence that *MTS2* or any other gene is involved. In contrast, the high frequency of point mutations and small deletions in *CDKN2* in melanoma primary tumours and cell lines further pinpoints *CDKN2* as a tumour suppressor gene involved in melanoma.

Apart from the present findings, the only indication that *CDKN2* may not correspond to *MLM* is based on the observation that somatic abberations in *CDKN2* are involved in diverse types of cancer cell lines, not only melanoma. Thus, predisposing *CDKN2* mutations might be expected to increase the occurrence of many cancer types in linked families. Indeed, some investigators have reported increased incidence of other tumours in melanoma-prone families[28] and it is unclear at present if 9p21-linked hereditary cancer is solely melanoma, or is truly multifocal.

The other possibility is that *CDKN2* and *MLM* are the same gene, but that predisposing mutations occur primarily outside the p16 coding sequence. This possibility is weakened by the observation of so many *CDKN2* coding sequence mutations in sporadic melanoma cell lines. In the subset of melanoma cell lines that do not contain *CDKN2* homozygous deletions, nearly half (14/34) contain nonsilent changes. If it is assumed that predisposing mutations are distributed in the same manner as somatic mutations, a similar percentage of the kindreds should have displayed mutations in the *p16* coding sequence. On the other hand, there is precedent for germline mutations falling outside the coding sequences of genes. For instance, in the thalassaemias a high percentage of germline mutations occur in noncoding regions that affect either protein translation, mRNA splicing or mRNA stability[29]; and an entire class of genes involved in neuronal development or function contains trinucleotide repeats outside the coding sequence that may predispose to disease[30] Moreover, it is conceivable that heterozygous, inherited, loss-of-function mutations in *CDKN2* might be lethal due to haploinsufficiency. In this case, predisposing *CDKN2* mutations might be expected to cause reduced expression of the gene and, therefore lie more frequently in sequences that regulate the level of *CDKN2* expression.

In summary, despite the appeal of *CDKN2* as a candidate

for *MLM*, we have encountered only two mutations in the p16 coding sequence which may confer susceptibility to melanoma. To prove or disprove the hypothesis that *CDKN2* is *MLM* will require an extensive search for predisposing mutations that lie in noncoding regions of the *CDKN2* gene or in neighbouring genes.

Methodology

MLM pedigrees and DNA samples. Lymphocytes were separated from whole blood using Ficoll-Hypaque (Pharmacia) according to the manufacturer's instructions. Lymphocyte DNA was extracted using standard procedures. Similar procedures were used to extract DNA from blood samples in the Netherlands and Australia.

PCR amplification and mutation screening. All three coding exons of *CDKN2* and their associated splice sites were amplified from tumour or matched normal genomic DNA using PCR[8]. The PCR conditions were: one cycle at 95 °C (5 min); 4 cycles at 95 °C (10 s), $T_{ann.}$ = 68 °C (10 s), 72 °C (10 s); 4 cycles with $T_{ann.}$ = 66 °C; 4 cycles with $T_{ann.}$ = 64 °C; 4 cycles with $T_{ann.}$ = 62 °C; 30 cycles with $T_{ann.}$ = 60 °C. The buffer conditions were as described except that 5% DMSO was added to the reaction[7]. The products were purified from 1.0% agarose gels using Qiaex beads (Qiagen) and analysed by cycle sequencing with α-^{32}p-ATP[31]. Products were run on 6% polyacrylamide gels. All adenosine reactions were loaded side by side, followed by the cytosine reactions, etc. Detection of polymorphisms was by eye with confirmation on the other strand. The DNA sequences of the primers used for *CDKN2* amplification and sequence determination were: exon 1, amplification: 1F – CAG CAC CGG AGG AAG AAA G; 1108R – GCG CTA CCT GAT TCC AAT TC; sequencing: 1108R; exon 2, amplification: 42F – GGA AAT TGG AAA CTG GAA GC; 551R – TCT GAG CTT TGG AAG CTC T; sequencing: 42F and 551R; exon 3, amplification 237F – CCA TTG CGA GAA CTT TAT CC; 654R – TGG ACA TTT ACG GTA GTGGG; sequencing: 237F and 654R.

Allele-specific oligonucleotide (ASO) analysis. PCR products were generated as described above and quantified after electrophoresis through 2% agarose gels plus ethidium bromide by comparison with known amounts of standard DNA. 10 µl PCR product was added to 110 µl of denaturant (7.5 ml, H_2O, 6.0 ml 1 N NaOH, 1.5 ml 0.1% bromophenol blue and 75 µl 0.5m EDTA) and incubated for 10 min at room temperature before blotting 30 µl on to Hybond membrane (Amersham) using a dot-blotting apparatus (GIBCO-BioRad). The DNA was fixed on the membrane by exposure to UV light (Stratagene). Prehybridization was carried out at 45 °C in 5× SSPE and 2% SDS[30]. Wildtype and mutant ASOs were labelled by incubation at 37 °C for 10 min in a reaction that included 50 µC_i γ-^{32}p-ATP, 100 ng ASO, 10 U T4 polynucleotide kinase (New England Biolab), and kinase buffer. 20 ng of labelled ASO was used in an overnight hybridization reaction in the same buffer as for prehybridization. Each blot was washed twice in 5×SSC and 0.1% SDS for 10 min at room temperature, followed by 30 min at progressively higher temperatures until nonspecific hybridization signals were eliminated. Blots were exposed typically for 40 min without an intensifying screen. The ASOs used are shown below. The wild type sequence is listed with the mutant base shown in parentheses following the base that it replaces in the wildtype sequence.

Base change location	Amino acid location	DNA sequence
436	140	AGA TGC CG(A)C GGA AGG
294	93	GGG CCG(T) GGG CGC
371	118	CGA TGT(A) CTC ACG GTA

Southern blots. 5 µg genomic DNA from each individual were digested with *Bam*HI or *Pvu*II and loaded onto a 0.7% agarose gel which was blotted according to standard procedures[31]. Cosmid c5 DNA was labelled by random hexamer priming[31]. Prehybridization, hybridization and filter washing were as described[32], except that 200 ng ml^{-1} total human DNA was added to the prehybridization reaction and incubated overnight.

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