Germ-Line Deletion Involving the INK4 Locus in Familial Proneness to Melanoma and Nervous System Tumors

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Abstract

Joint predisposition to malignant melanoma and nervous system tumors (NSTs) is a puzzle. Several melanoma susceptibility genes have been identified, including p16, a clustered tumor suppressor. However, the molecular bases of inherited proclivity to NSTs in the absence of a recognizable genetic syndrome are unknown. We analyzed two families with joint proneness to melanoma and NSTs in view of genetic linkage and identification of the causal molecular lesions. Highly informative linkage markers were used for segregation analyses of the predisposition alleles in the two pedigrees. Characterization of the molecular lesions required hemizygosity mapping based on microsatellite markers physically mapped to contigs of the 9p21 region and a Southern blot approach using several PCR-generated probes. Both families were found to be allelic and linked to p16 markers. In the family segregating the melanoma/NST syndrome, a large germ-line deletion ablated the whole p16, p19, and p15 gene cluster (or INK4 locus), whereas a more circumscribed molecular lesion disrupting p16 and p19 but leaving p15 unaltered segregated with the melanoma-astrocytoma syndrome (MIM 155755). Our results suggest that multiple cancer susceptibility in these two families ensues from contiguous tumor suppressor gene deletion. Indeed, known phenotypes associated with germ-line p16 mutations and an apparent correlation between the deletion span and tumor spectrum in the two families suggest a new model of cancer pathogenesis based on the inactivation of contiguous tumor suppressor genes, an alternative to the established pleiotropic effects of single-gene disruption.

Introduction

Familial clustering of NSTs has been demonstrated in several family- and population-based surveys, suggesting segregation as a mendelian disorder (1). Among the possible pathological findings are astrocytoma/glioblastoma, meningioma, ependymoma, and medulloblastoma, reflecting the overall heterogeneity of these familial conditions.

CMM is a well-established tumor predisposition condition sometimes associated with precursor lesions in familial atypical multiple mole melanoma or dysplastic nevus syndrome (2). One of the major CMM loci has been mapped in 9p21, with the consecutive identification of mutations in the p16 gene (also termed CDKN2A, INK4a, or MTS1; see Ref. 3 for a review), which encodes a CDKN protein (P16). However, some of the 9p21-linked pedigrees lack an identifiable mutation in p16. Another CMM locus has been defined in 1p36 that contains an as yet unidentified susceptibility gene (4). Families with dysplastic nevus syndrome are primarily linked to the 1p36 locus, although some were found to segregate with 9p21 markers. Finally, a single activating missense mutation of CDK4 accounts for a small fraction of the families that demonstrate proneness to CMM (5). This mutation makes the encoded protein insensitive to inhibition by p16 and defines a third CMM locus in 12q13. None of these families has been associated with NSTs.

Following the observation that patients with NSTs are at higher risk of developing CMM, a large population-based survey performed in Israel demonstrated increased risk of NSTs in melanoma patients as well as in their first- or second-degree relatives (6). The authors suggested joint predisposition segregating as a mendelian disorder.

We have described a familial cancer syndrome of CMM and NSTs consistent with an autosomal dominant mode of inheritance (7). Pathological findings of CMM, DN, astrocytoma, neurofibroma, schwannoma, and meningioma indicated strong overlap with the NF1 tumor spectrum (family A; Fig. 1a). However, mutation screening in the NF1 gene and segregation analyses using highly polymorphic intragenic markers precluded allelism of this familial syndrome to NF1 (7). Another family showing segregation of CMM and NSTs served as a basis for the report of a novel CMM-astrocytoma syndromal entity (melanoma-astrocytoma syndrome, MIM 155755; Ref. 8; family B; Fig. 1b). Herein, we present evidence for allelism of the two CMM-NST syndromes to the INK4 locus as the result of a contiguous genetic lesion. A possible correlation between the deletion span and tumor spectrum suggests a specific role for different genes in defining proneness to CMM or NST subsets or, in other words, their involvement in familial NSTs in the absence of proclivity to CMM.

Patients, Materials, and Methods

Patients. Two families exhibiting clustering of CMM and NSTs were studied for the mapping and molecular characterization of a putative joint predisposition allele to tumors of both systems.

Family A (Fig. 1a) has been investigated since 1977 at the Hôpital Pitié-Salpêtrière (Paris, France). About 100 individuals were surveyed, and pieces of pathological material initially investigated in 16 different laboratories were, when possible, reassessed by both a dermatopathologist and a neuropathologist. Extensive historical and clinical descriptions of this kindred are reported elsewhere (7). Overall, six members of this family are or were affected by CMM, and seven members are or were affected by NSTs, ranging from astrocytoma of all grades to schwannoma, neurofibroma, and meningioma. Four of the relatives have both skin tumors and NSTs, with an additional two if considering individuals with obligate carrier status, resulting in statistically significant cosegregation of the skin tumor and NST traits. Therefore, this
family illustrates a melanoma/NST syndrome inherited as a single autosomal dominant mendelian trait.

Family B (Fig. 1b) has been investigated at the Mayo Clinic (Rochester, Minnesota) since 1992. An extensive historical, clinical, and neuropathological description of this kindred has been reported elsewhere (8). Briefly, seven members of this family are or were affected by CMM, and three members are or were affected by primary glial tumors, including two grade 4 astrocytomas and one cerebellar astrocytoma. Four of the other family members had other malignancies (lung cancer in a 70-year-old man, leukemia of an unspecified type in a woman in his 70s). Like the French kindred, this family illustrates a melanoma/NST syndrome inherited as a single autosomal dominant mendelian trait, possibly including additional oncological manifestations.

Microsatellite Typing. A series of 9p21-linked microsatellite markers were typed for segregation analyses in these two families. Their locations are shown in Fig. 2. All primer oligonucleotide sequences are available through the Genome Data Base (http://gdbwww.gdb.org). After amplification with a fluorescein 5'-end-labeled primer, followed by the addition of an internal size standard (GeneScan 2,500-ROX; PE Applied Biosystems), PCR products were separated on a 6% polyacrylamide/7M urea gel using an ABI PRISM 377 DNA sequencer (PE Applied Biosystems). Analysis was performed using the GeneScan 672 (version 1.2) software package.

Sequence Analyses. Nucleotide sequences were determined from genomic DNAs, which were prepared according to standard procedures from either freshly isolated leukocytes or EBV-transfected lymphoblastoid cell lines, using the ABI PRISM dye terminator cycle sequencing ready reaction kit (PE Applied Biosystems) with AmpliTaq DNA polymerase FS. following the manufacturer's specifications. The p15, p19, and p16 gene exons were analyzed using the PCR primer pairs determined by Jen et al. (Ref. 9; see "Discussion" and Fig. 2 for a description of the INK4 locus). Finally, the extension products were analyzed with an ABI PRISM 377 DNA sequencer.

Southern Blot Analyses. A HindIII digest of 12 μg of high molecular weight genomic DNAs from patients and controls was prepared for Southern blot analyses following standard procedures. Three probes were generated by PCR using primers p15-2F and R2.7R (probe 1, ~8.4 kb), R2.7F and E1β-RB
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![Physical and deletion map of the 9p21 region.](image)

Fig. 2. Physical and deletion map of the 9p21 region. p15, p19, and p16 gene exons are indicated by rectangles, and their respective primary transcripts are indicated by arrows, in agreement with Cayuela et al. (36). Microsatellite markers D9S976, D9S975, D9S1748, D9S942, D9S958, D9S974, and D9S736 are physically positioned from the centromere to the 9p telomere: the scale represents 3 kb, except for double bars indicating gaps of variable lengths. Southern blot probe 1 spans p15 exon 2 through STS R2.7, probe 2 spans STS R2.7 through p19 exon 1β, and probe 3 spans p19 exon 1β through STS R2.3, all meant to hybridize to a wild-type ~17-kb HindIII fragment, as indicated by the positions of the relevant restriction sites. The mutant alleles are symbolized for family A and family B: open bars indicate that the fragment is retained (not deleted), and closed bars indicate that the fragment is deleted. The deletion in family A spans the whole gene cluster, whereas the centromeric breakpoint in family B lies within p19 exon 1β, sparing p15 but ablated the whole p16 sequence. These large molecular lesions segregate with the boxed haplotype in Fig. 1a or b.

Results

Segregation Analyses. After exclusion of several candidate genes or loci, the recently described melanoma/NST syndrome was found to be consistent with linkage to highly polymorphic markers in 9p21. Indeed, all individuals who had developed CMM, NST, or both shared a common haplotype at this locus, as defined by markers IFN1@, D9S1845, D9S976, D9S1748, and D9S169 (Fig. 1a). Possible linkage was considered, given the hypothesis of nonpenetrance in some of the family members and phenocopies in those previously thought to be affected on the basis of nonspecific dermatological findings. Because of a high mortality rate, fewer meioses were available for analysis in family B (Fig. 1b). However, segregation analyses still indicated possible linkage to 9p21, using a different set of markers (IFN1@, D9S736, D9S1749, D9S171, and D9S126) selected for reasons of informativeness (Fig. 1b).

Sequence Analyses. To determine whether a point mutation of p15, p16, or both segregated in either or both families, nucleotide sequences of the two CDKN genes were established from genomic DNAs of patients and their unaffected relatives or unrelated controls. No genetic change was observed in either pedigree (data not shown).

Hemizygosity Mapping. In keeping with the identification of a putative rearrangement at this locus, a microsatellite marker lying 3' to p19 exon 1β (D9S1748; Ref. 12) was studied by segregation analyses and found to be deleted in the two families (Figs. 2 and 3). The definition of the molecular lesion was refined in each family using a series of polymorphic markers, based on evidence of hemizygosity. Because of the tentative positioning of linkage markers in the different on-line integrated maps, only physically positioned markers are presented here. These include (from centromere to telomere) D9S976, D9S975, D9S1748, D9S942, D9S958, D9S974, and D9S736 (13). In family A, the centromeric deletion breakpoint lies between markers D9S976 and D9S975, whereas the telomeric breakpoint lies between markers D9S974 and D9S736, thereby defining a large region encompassing the whole INK4 locus (Fig. 2). In family B, the centromeric deletion breakpoint lies between markers D9S975 and D9S1748, whereas the telomeric breakpoint lies between markers D9S974 and D9S736 (Fig. 2), leaving the position of the centromeric breakpoint uncertain with regard to p15.

Southern Blot Studies. To refine the location of the centromeric breakpoint in family B, a series of Southern blot probes was generated by PCR. Using this approach, p15 was found not to be deleted in family B, as demonstrated by the presence of a 16-kb HindIII junction fragment using probes 1 and 2 (Fig. 2), as compared with the wild-type 17-kb allele (Fig. 4). The presence of a faint 16-kb band still detected with probe 3 (data not shown) indicates that the centromeric breakpoint lies within p19 exon 1β (Fig. 2), thereby defining a ~100–200-kb deletion span.

Discussion

Evidence for cosegregation of the tumor predisposition traits with large DNA deletions involving the INK4 locus in each pedigree mandates further definition of the molecular structure and transcriptional regulation of this complex genetic region, as well as of the putative functions of the different encoded proteins. The p16 gene is composed of three exons, namely exons 1α, 2, and 3, and encodes a cell cycle inhibitor protein that, through its ability to specifically bind cyclin kinases CDK4 and CDK6, hinders phosphorylation of the
retinoblastoma protein (pRB) by cyclin D/CDK complexes and entry from G1 into S phase (14). A certain level of complexity results from the fact that another cell cycle inhibitor protein is encoded by the overlapping p19ARF gene (p19), composed of its proper upstream exon 1ß and of a p16-shared exon 2 (Fig. 2). p19 exhibits no in-frame homology with p16, and its protein product is not a direct CDKN. However, the overexpression of p19 leads to cell cycle arrest (15), probably through a pathway that leads to the activation of multifunctional p53 (16). About 30 kb centromeric of p16 lies p15 (also termed CDKN2B, INK4b, or MTS2; see Ref. 17; Fig. 2), which shares a strong homology with p16 in the 5' two-thirds of exon 2, and whose protein product also binds CDK4 and CDK6.

A large body of data concerns the somatic events that affect the INK4 locus during cancer pathogenesis. The disruption of p16 is observed in a large array of tumor cell types, explaining its being referred to as a major tumor suppressor gene. Down-regulation at the somatic level results from loss of heterozygosity, point mutations, or gene silencing (3). Interestingly, p16, or the P16/cyclin D/CDK4/pRB pathway, is primarily altered in melanoma pathogenesis (18, 19), in which loss of p16 expression correlates with tumor progression (20), as well as in glioma lesions (21, 22). However, germ-line mutations disrupting p16 have been primarily associated with CMM (with or without DN) and secondarily associated with epithelial malignancies such as carcinoma of the pancreas (23) or squamous cell (24) carcinomas, but they have never been identified in families with NSTs.

With regard to p19, all reported germ-line mutations reside in...
p16-shared exon 2. Phenotypes do not substantially differ from mutations of p16 that do not affect the p19 coding sequence (25). More interestingly, cancer-associated missense mutations of exon 2 that impair the ability of P16 to induce cell cycle arrest do not alter G1 or G2 arrest by mutant P19 proteins (26). These results suggest that the functional domain of P19 is encoded by the specific exon 1B, or that mutations of p19 are inconsequential to cancer pathogenesis. However, there has been renewed interest in the role of this gene due to the recent ablation of exon 1B in the mouse by homologous recombination. Quite surprisingly, young p19 null mice develop carcinomas, fibrosarcomas, and hematological malignancies either in a spontaneous manner or on exposure to a carcinogen or radiation (16). Because the p16 gene is still functional in p19 knockout mice (27), it is of interest to examine p15 and/or p19 in humans with inherited predisposition to cancer, especially in families that segregate CMM (31) or NSTS (32).

It is difficult to assign a specific role to p15, p16, or p15 in tumor progression, because the three of them are often concomitantly altered at the somatic level by either loss of heterozygosity or gene silencing. Inactivation of all three genes has an epistatic, i.e., synergistic, effect. Whatever the case, it will be of interest to examine p15 and/or p19 in humans with inherited proneness to NSTS in the absence of proclivity to CMM.

References


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