Allelotypes of Primary Cutaneous Melanoma and Benign Melanocytic Nevi

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ABSTRACT

A multistep genetic model of tumorigenesis, based on genetic alterations in benign and primary malignant lesions, has been proposed for neoplasms such as colonic carcinoma. However, evidence for a similar genetic progression in melanoma has relied heavily on findings in cultured lesions or metastases. We have investigated every autosomal arm for loss of heterozygosity in 41 primary cutaneous melanomas and 32 benign melanocytic nevi, and have investigated several chromosome arms that show loss in melanoma in 27 Spitz nevi (a nevus with histological similarities to melanoma). Loss of heterozygosity in primary melanoma was identified most frequently on chromosomes 9p (46%) at loci near the p16INK4a gene, 10q (31%), 6q (31%), and 18q (22%); loss of these chromosome arms were related to the progression of the melanoma. Only two benign melanocytic nevi (both of which showed atypical features on histology) demonstrated genetic alterations, including 9p loss in one case. In addition, two Spitz nevi contained interstitial deletions on chromosome 9p. Our findings show that loss of heterozygosity of 9p is not confined to melanoma, but that other uncultured melanocytic lesions can also display loss of this chromosome arm, and that other genetic changes (e.g., loss of 10q, 6q, and 18q) may be important in conveying the malignant phenotype to melanoma.

INTRODUCTION

The incidence of cutaneous melanoma is increasing worldwide, and because metastasis may occur early in this disorder, treatment is often unsatisfactory (1–5). An understanding of the early genetic changes in melanoma tumor evolution may, therefore, lead to improvements in therapy. In other cancers, notably colorectal cancer, tumorigenesis proceeds via a multistep process in which there is a causal relation between the accumulation of genetic abnormalities and incremental changes in clinical behavior (6–8). In melanoma, studies to date have implicated tumor suppressor genes in its pathogenesis, as well as mutations of oncogenes such as ras (9–11); however, most studies of genetic losses have relied on examination of cell lines, cultured primary melanocytes, or metastases, rather than examination of primary tumor material (12–22). Karyotype analyses of nevi and melanomas have identified structural abnormalities of multiple chromosomes, including chromosomes 1, 6, 7, 9, and 11, but results vary between studies (20–22). LOH in cutaneous melanoma has been identified on chromosomes 9p (12–14, 23), 1p (15, 23), 6q (16, 17, 23), 11q (18, 23), and 10q (19, 23, 24), although for the majority of areas showing LOH, candidate tumor suppressor genes are not known. Recent evidence suggests that the p16INK4a gene located at 9p21 is important in sporadic and familial melanoma pathogenesis (25–33), but deletion-mapping studies suggest that other tumor suppressor genes on 9p may also be involved (13, 25, 34). Because no full allelotype of LOH in melanoma or nevi has been published to date, the relative importance and contribution of loss of individual chromosome arms to melanoma development is unclear. In addition, one possible problem that arises with the use of cultured material is that genetic alterations may have occurred secondary to the tissue culture process, and results may not be representative of the in vivo situation. LOH in metastatic melanomas may also reflect secondary or late genetic events that are not relevant in the development of the primary lesion.

Studies defining the clinicopathological relation between melanoma and other melanocytic lesions have been hampered by the fact that it is impossible to know the histology of a lesion and its continuing biological behavior. Thus, some consider that most melanomas arise directly from normal melanocytes (35), others that melanomas frequently arise in preexisting melanocytic nevi (36). Similarly, dysplastic nevi are thought by some to be precursors of melanoma (36–38), by others merely risk factors for melanoma development in susceptible individuals (35); the relation between germline p16INK4a mutation and dysplastic nevi appears weaker than that seen for p16INK4a mutation and familial melanoma (28). It has also been suggested that melanomas initially proceed through a lateral growth phase that is incapable of metastasizing (39) to a vertical growth phase in which metastasis can occur. However, the best single prognostic factor for invasive melanoma is the Breslow thickness [i.e., the depth of invasion of the primary tumor as measured from the granular layer of the epidermis (4, 40)]; primary tumor thickness presumably acts as a proxy for the ability of the primary tumor to metastasize, which in turn reflects the accumulation of genetic abnormalities.

The finding of genetic changes in a tumor does not prove that these alterations are causally implicated in the behavior of the tumor. For this reason, it is important to examine other melanocytic lesions rather than just melanomas. Spitz nevi (juvenile melanomas) are melanocytic tumors that most commonly occur in childhood (41). Despite sharing several histological features with melanoma, including, in some instances, nuclear atypia and occasional anecdotal reports of subsequent metastases and lymphatic invasion, the vast majority of Spitz nevi are benign and are managed accordingly (41–43). The relevance of any genetic changes found in melanoma in terms of explaining clinical behavior may, therefore, be qualified by examination of Spitz nevi. In this study, we have carried out an allelotype of 41 uncultured primary cutaneous melanomas and have investigated 27 Spitz nevi for LOH on several chromosome arms in an attempt to determine the importance of changes found in melanoma. In addition, because other workers have suggested that LOH of 9p and/or 10q is likely to be present at the common acquired nevus stage of melanoma development (44, 45), we have also carried out an allelotype of 32 benign melanocytic nevi.

MATERIALS AND METHODS

DNA Extraction. Genomic DNA was extracted from paraffin-embedded samples in all cases, including 41 sporadic primary cutaneous melanomas, 27
Spitz nevi, and 32 benign melanocytic nevi, by the phenol chloroform method and ethanol precipitation (46). To ensure that none of the Spitz nevi were primary cutaneous melanomas, all cases that were originally histologically and clinically diagnosed as Spitz nevi were reexamined by a second pathologist with expertise in the interpretation of melanoproliferative lesions (H. R.) before inclusion in the study. All specimens were initially microdissected to reduce contamination with normal tissue and subjected to SIZ/protease K digestion for 5 days at 37°C. DNA from normal skin or peripheral blood was used as control in each case. Elutip-d chromatography was used to further purify deeply pigmented tumors, to prevent coextracted melanin from inhibiting the PCR (19).

Allelotyping. Normal and tumor DNA were amplified using microsatellite primer oligonucleotides obtained from Research Genetics (Huntsville, AL; Table 1). Standard PCR was carried out in a total of 10 μl containing 100 ng of genomic DNA, 200 mm concentrations of each dNTP, 1 pmol of each primer (one end-labeled with [γ-32P]ATP), and 1 unit of Taq polymerase (BioTaq; Bioline, London, United Kingdom). Amplifications were done in a Perkin Elmer Cetus DNA thermal cycler Model 480 and consisted of 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, with an extension time of 10 min at 72°C during the final cycle. The products were electrophoresed through 6% denaturing polyacrylamide gels, which were subsequently dried and exposed to Fuji XR film for 1–48 h. Allelic loss was identified from informative cases from a significant reduction of one allele in the tumor DNA as compared with the control DNA.

RESULTS

A panel of highly informative microsatellite markers was used to screen 41 primary cutaneous melanomas and 32 benign melanocytic nevi for LOH on every autosomal arm (Table 1). LOH in primary melanomas was identified most frequently on chromosome 9p (46%) at loci close to the p16INK4a gene, but LOH also occurred commonly on chromosomes 10q (31%), 6q (31%), and 18q (22%) (Fig. 1). In contrast, LOH of these chromosome arms was not detected in 31 of the 32 benign nevi. However, loss of 9p at D9S162 and D9S171 was seen in one benign melanocytic nevus (N6) that displayed some cytological atypia, lentiginous proliferation, and occasional intraepidermal spread of nevus cells. In an attempt to identify whether the 9p loss was restricted to one area within this nevus, we further microdissected the specimen into four subsections before DNA extraction and microsatellite analysis. LOH of 9p was not confined to any area within the nevus but was detected in each of the four parts (Fig. 2). Investigation of LOH in 27 Spitz nevi with markers that showed frequent loss in the melanoma group, including several microsatellite markers on 9p (Table 2), resulted in an interstitial deletion of chromosome 9p being identified in two instances [loss at D9S162 and retention at D9S168 and D9S171 in one case (JN20), and loss at D9S171 and retention at D9S162 and D9S176 in the other case (JN50)]; no loss was seen at any other locus in either of these or in the other Spitz nevi. Unfortunately, direct cycle sequencing of the p16INK4a gene in the melanomas gave stop bands at multiple sites in all four lanes as a result of the particular fixation protocol used, despite it working in blood and frozen samples, precluding attempts to identify mutations within this gene in the melanomas and other lesions showing LOH at this locus (data not shown).

In addition to 9p loss occurring in some benign lesions, LOH of 9p was detected in 3 of 9 informative superficial spreading melanomas that had invaded less than 1.5 mm in depth, as well as in deeper lesions. However, although 10q loss was also detected in melanomas of less than and greater than 1.5 mm in depth, no benign lesion demonstrated loss of 10q. In contrast, allelic losses of 6q and 18q were detected only in thicker lesions that had invaded deeper than 1.5 mm. LOH ranged from 0 to 19% on the other 35 autosomal arms, although it is possible that some of the allelic imbalances detected represent amplification rather than loss at the locus investigated. It was of interest that 5 of 10 melanomas that had invaded less than 1.5 mm in depth demonstrated loss of other chromosomes besides 9p and/or 10q: (p+q) loss in one case, LOH of 3p and 10p (in addition to 10q loss) in another case, 8q and 21q loss in a third, LOH of 14q in a fourth, and 19p loss in a fifth. LOH of other autosomal arms, besides 9p, was not observed in any of the benign nevi, apart from one nevus (N18) that not only demonstrated allelic losses at 8p, 11q, and 12p but also showed band shifts at multiple loci. It was of interest that although this nevus was benign, there was some cytological atypia, lentiginous proliferation, and lamellar fibroplasia; however, the lesion did not fulfill strict criteria for designation as a dysplastic nevus. Single band shifts at isolated loci were detected in 7 primary melanomas, but no primary melanoma demonstrated evidence of a replication error repair defect being important in its development (i.e., multiple band shifts at any locus or band shifts at more than one locus). Similarly, two Spitz nevi contained band shifts at isolated loci; neither of these lesions demonstrated LOH at any locus.

DISCUSSION

We have identified loss of chromosome arms 9p, 10q, 6q, and 18q as the most frequent genetic losses in an allelotype study of primary cutaneous melanomas but have detected LOH infrequently in benign melanocytic nevi and in Spitz nevi. None of the lesions (melanomas
or nevi) included in the study was taken from patients with atypical mole syndrome. The majority of benign melanocytic nevi were removed for cosmetic purposes, and all were both clinically diagnosed and histologically confirmed as benign. The finding of loss of 9p, 10q, and 6q in our primary melanomas confirms the results of previous LOH investigations on cell lines and metastases (12–14, 16, 17, 19–22) and adds further support that tumor suppressor genes on these chromosome arms are important in cutaneous melanoma development. The observation of 18q loss in 22% of primary melanomas suggests that one of the several tumor suppressor genes relevant in melanoma is located on this chromosome arm; a potential candidate would be the DCC gene in view of its high expression in neural crest-derived cell lines (48). In addition, our results provide an explanation for the differences in the clinical behavior between melanomas and the benign lesions studied, in that loss of 10q, 6q, or 18q was not identified in any of the benign lesions. Furthermore, the identification of LOH and/or single band shifts in two nevi and in four of the Spitz nevi (from six different individuals) demonstrates that these lesions in vivo are clonal in nature.

The detection of LOH of 9p in a benign but atypical melanocytic nevus, as well as frequent loss of 9p in primary melanomas (including melanomas that have invaded <1.5 mm in depth), all point to 9p loss as an early event in the pathway of progression to melanoma. However, in contrast to previously proposed genetic models for melanoma progression (44, 45), neither 9p nor 10q loss was detected in 30 benign melanocytic nevi that were entirely typical histologically, suggesting that LOH of these chromosomes is not involved in the development of a common acquired nevus from a normal melanocyte. The finding of 9p loss in Spitz nevi and a benign atypical nevus also suggests that 9p loss is not sufficient for melanoma initiation, in accordance with recent work showing that loss of p16INK4 expression occurs after tumor initiation, and with the absence of melanoma in an individual harboring homozygous germline p16INK4 mutations (30, 33).

In addition to 9p loss, LOH of 10q was detected in melanomas that had invaded less than 1.5 mm deep, suggesting that loss of 10q is the next genetic event that occurs frequently in melanoma development. Because LOH of 10q was not detected in any of the benign lesions but is a relatively early event in melanoma progression, it may be that the disruption of function of the relevant tumor suppressor gene on this chromosome arm is the genetic event that conveys the malignant/invasive phenotype to the melanoma; however, further

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<th>Chromosome arm</th>
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<tr>
<td>3p</td>
<td>D9S1293</td>
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</tr>
<tr>
<td>6q</td>
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<td>18q</td>
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*Markers and marker position relative to selective references from the CEPH database are as described by Weissenhoch et al. (47). Results shown are number of tumors showing LOH/number of informative cases for one or more markers on each chromosome arm.

**Microsatellite markers are listed in order from 9pter to 9qter.**

Table 2: Frequency of loss of heterozygosity of microsatellite markers on several chromosome arms in microdissected Spitz nevi

Fig. 1. Frequency of allelic deletions on individual chromosome arms in primary cutaneous melanomas. □ p arm; ■ q arm. Chromosomes examined are indicated on the X axis.

Fig. 2. Allele losses on chromosome arm 9p in a benign melanocytic nevus with atypical features, a Spitz nevus, and two primary cutaneous melanomas. Lanes 1 and 6, control DNA from subject with benign melanocytic naevus with atypical features; Lanes 2–5, DNA from four separate areas of same benign melanocytic nevus with atypical features showing LOH at D9S162 in all four areas; Lane 7, control DNA from subject with Spitz nevus; Lane 8, DNA from Spitz nevus showing LOH at D9S162. Lanes 9 and 11, control DNAs from two subjects with primary cutaneous melanoma; Lanes 10 and 12, DNA from corresponding primary cutaneous melanomas showing LOH at D9S171.
work will be necessary to substantiate this hypothesis. The finding of allelic losses at other loci in melanomas that had invaded less than 1.5 mm in depth is also interesting; it suggests that some melanomas arise through alternative pathways as a result of these chromosome losses, or that secondary genomic instability can result at an early stage in melanoma invasion. LOH of chromosomes 6q and 18q occurred relatively frequently but were limited to primary melanomas that had invaded deeper than 1.5 mm. Previous models on genetic progression in melanoma have proposed the involvement of a gene on 6q in the radial growth phase of primary melanoma (44, 45), but our data are more consistent with loss of 6q occurring in thicker lesions, suggesting that it (and loss of 18q) is more likely to be involved in the subsequent development of metastases. Further support for this comes from the evidence provided by Welch et al. (49), using microcell-mediated chromosome transfer, for the location of a melanoma metastasis-regulatory gene on human chromosome 6.

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