Loss of Heterozygosity in the *MXI1* Gene Is a Frequent Occurrence in Melanoma

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Melanoma development and progression is thought to be the result of a multi-step accumulation of genetic damage, with loss of heterozygosity in chromosome 9p (*MTS1*) frequently described. In addition, chromosome 10q allelic loss has been reported, implicating the tumor suppressor gene *PTEN/MMAC1* on 10q23.3. The *MXI1* gene at 10q24–25 is another candidate tumor suppressor that has only rarely been studied in melanomas, with conflicting results. We used microdissection-based genotyping to investigate 29 melanomas from 20 patients for loss of heterozygosity in intragenic and flanking microsatellite markers for this latter gene. Concurrently, the *MTS1* gene was similarly studied using two flanking microsatellites. Fifty-four percent (15 of 28) of the informative cases showed loss of heterozygosity for one or both *MXI1* markers, as compared with 67% (16 of 24) of the informative cases for *MTS1*. *MXI1* allelic loss was seen more frequently in recurrent/metastatic tumors (59%), as compared with in primary (33%) lesions. Eighty percent of the primary tumors showed loss of heterozygosity for *MTS1*, as well as 63% of recurrent/metastatic ones. We studied more than one tumor in eight patients, with those from three patients showing discordant genetic patterns. One patient showed a metastatic tumor with allelic loss for *MXI1* that was not identified in the primary melanoma or a local recurrence. The other two patients showed clonal heterogeneity in *MXI1* at synchronous and metachronous metastatic foci. These findings support *MXI1* as a putative tumor suppressor gene involved in conventional melanoma progression. Genetic heterogeneity seen in different metastases from the same primary suggests a non-linear pattern of chromosomal damage, with the development of multiple clones within the primary tumor, each acquiring its own metastatic potential.

KEY WORDS: Clonal diversity, Loss of heterozygosity, Melanoma, *MTS1/CDKN2A*, *MXI1*.

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Melanoma is a significant health concern among the Caucasian population and is typically characterized by aggressive biologic behavior, with eventual metastasis and death. Tumorigenesis is thought to be associated with a multistep, temporal accumulation of genetic alterations (1), and in an attempt to understand its biology, the molecular pathogenesis of these lesions is being continuously investigated. Of the chromosomal abnormalities encountered, allelic imbalances of chromosome 9p21, which harbors the *MTS1* gene are those frequently described in both sporadic and familial melanomas (2–7). Mutations involving the long arm of chromosome 10 have also been reported in sporadic primary and metastatic melanomas at frequencies ranging from 31 to 58% (4, 5, 8–11) and have been associated with a poor prognosis (4, 8).

The Max interacting protein (*MXI1*) is a tumor suppressor gene mapped to chromosome 10q24–25. Deletion of genetic material from this site is commonly seen in neural crest–associated tumors such as glioblastomas (12). Rao *et al.* (11) demonstrated *MXI1* loss of heterozygosity (LOH) in 46% of the primary and metastatic desmoplastic melanomas that they studied, postulating that the embryologic neuroectodermal origin of desmoplastic melanomas may account for this similarity with glioblastomas. Extrapolating on this further, it would not be surprising if conventional melanomas should show similar allelic imbalances in the *MXI1* gene. Herbst *et al.* (13), however, in their study of 65 sporadic primary and metastatic melanomas, found only 7 to 13% LOH for this gene. To our knowledge, these are the only two published studies in the English literature on *MXI1* in melanomas.

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We thus sought to further investigate the role of the *MXI1* gene in the pathogenesis and progression of conventional melanomas by using a microdissection-based genotyping technique to look for LOH using two microsatellite markers for this gene. We compared these findings to those seen in the *MTS1* gene on 9p21.

MATERIALS AND METHODS

Seven primary, 2 recurrent, and 20 metastatic melanomas were identified from the files of the Presbyterian and Montefiore University Hospitals of the University of Pittsburgh Medical Center. Paraffin-embedded blocks were obtained for each of the 29 cases, and clinical information was extracted from the Medical Archives Retrieval System.

Serial 4-μm-thick unstained histologic sections were microdissected under stereoscopic observation, as described elsewhere (14). For each tumor sample, representative uninvolved dermis of approximately equal size was also obtained as a normal control. The microdissected samples were treated with 50 μL of proteinase K (10 ng/mL) for 2 hours and boiled for 5 minutes to remove enzyme activity.

Each sample was PCR amplified in individual reactions using oligonucleotide primers targeted at microsatellite repeats of the *MXI1* and *MTS1* genes. For *MXI1*, we used an intragenic microsatellite (3NT) in the 3' nontranslating region of the gene (12), and the flanking repeat unit *D10s1173* (GenBank accession no. L30341). *MTS1* was studied using two flanking microsatellites *D9s251* and *D9s254* (GenBank accession nos. L18726 and L18050, respectively). The primers for *MXI1.3NT* were 5'-GGTTACTCCAGTGCCAGTGT-3' (sense) and 5'-TTAAATACAGGTCCTCTGACCC-3' (antisense); for *D10s1173,* they were 5'-CATGCCAAGACTGAAACTCC-3' (sense) and 5'-AAACCCCAATGCCATAATGG-3' (antisense); for *D9s251,* they were 5'-CCTGTGTTGAAATTTTGACTG-3' (sense) and 5'-ATTTCAGACTTCCTTGTGTTC-3' (antisense); and for *D9s254,* they were 5'-TCCTGGGTAATAACTGCCG-3' (sense) and 5'-CACTCACACACACGCTCAG-3' (antisense). After initial denaturation at 95° for 10 minutes, 35 cycles of PCR were carried out with denaturation at 94° C for 1 minute, annealing at 58° C for 1 minute, and polymerization at 74° C for 1 minute, with a final 10-minute extension at 74° C. The amplified products were labeled with [P^{33}]-deoxyadenine triphosphate and electrophoresed in 6% polyacrylamide. Loss of heterozygosity was determined by comparing the intensity of the polymorphic bands from normal and tumor tissue. To control for allelic dropout caused by insufficient sample size, the bands from normal tissue were required to be of similar intensity to justify analysis of the tumor samples.

RESULTS

Twenty patients were identified with 7 primary, 2 recurrent and 20 metastatic melanomas (Table 1). Overall, both genes showed similar degrees of allelic loss, 54% for *MXI1* and 67% for *MTS1*. For the *MXI1* gene, comparing primary and recurrent/metastatic lesions, 6 and 22, respectively were informative for one or both markers. LOH was seen in 2 (33%) and 13 (59%) of these (Fig. 1). Similarly analyzing *MTS1*, 5 and 19 of primary and recurrent/metastatic tumors were informative, with 4 (80%) and 12 (63%) showing LOH, respectively.

More than one tumor was sampled in eight patients. Of these, the pattern of LOH in both genes was identical in five. One of the discordant cases (Patient 1) was that of a primary melanoma of the left leg that was negative for LOH in *MXI1* and recurred 6 months later with a similar result. The metastasis from 1 year later, however, showed acquisition of LOH in both loci of the *MXI1* gene (Fig. 1). *MTS1* was not informative. Patient 10 had two distinct metastases sampled within 1 week of each other. The adrenal tumor showed LOH in all four gene loci studied, whereas the lung was positive in only one locus for each gene (*MXI1.3NT* and *D9s251*) and was negative in the other two. The primary tumor, a trunk melanoma from 19 years prior, was not available for analysis. The results from Patient 11 were of particular interest, with the two tumors studied representing neck lymph node and parotid gland metastases that were removed 6 and 8 years, respectively, after a right cheek primary. Although on initial inspection the pattern of gene loss appeared to be the same for both lesions, examination of the allelic bands lost for the *MXI1.3NT* microsatellite revealed that different ones were deleted in the separate metastases and thus represented genetically distinct clones (Fig. 1).

DISCUSSION

Genetic alterations involving the long arm of chromosome 10 occur fairly commonly in primary and metastatic melanomas, with frequencies ranging from 31 to 58% (4, 5, 8–11). The gene most often implicated is *PTEN/MMAC1* (9, 10), a tumor suppressor gene located on 10q23.3. Germline mutations of this gene have been associated with Cowden disease (multiple hamartoma syndrome), a rare autosomal-dominant familial cancer syndrome (15). The *MXI1* gene is also located on the long arm of chromosome 10 and has been mapped to chromosome 10q24–25. This latter gene acts as a tumor suppressor by negatively regulating the myc oncoprotein. Allelic imbalances have been demonstrated in glioblastomas (12); however, to our knowledge, there have been only two prior studies

TABLE 1. Loss of Heterozygosity in Melanomas for *MXI1* and *MTS1* Using Two Microsatellite Markers for Each Gene

Patient	Age	Sex	Sample Site	Sample	TNM	Breslow Depth (mm)	Clark's Level	MXI1		MTS1	
								3NT	D10s1173	D9s251	D9s254
1[a]	57	F	Left leg	P	ND	ND	ND	−	−	NI	NI
			Left leg	R	N/A	N/A	N/A	−	−	NI	NI
			Groin lymph node	Me	N/A	N/A	N/A	+	+	NI	NI
2	71	M	Back	P	pT4aN0Mx	5.0	IV	NI	−	NI	NI
3	42	M	Left back	P	pT3bN1Mx	4.0	IV	+	NI	+	+
			Left axilla LN	Me	N/A	N/A	N/A	+	NI	+	+
4	63	M	Right upper arm	P	pT4aNxMx	6.5	IV	NI	NI	NI	+
5[b]	46	M	Left waist	P	pT3aNxMx	2.68	IV	−	−	+	+
6	28	M	Scalp	P	pT3aNxMx	2.87	IV	−	−	+	−
7	41	F	Left leg	P	PT2NxMx	0.49	III	NI	+	−	−
8	46	M	Shoulder	Me	N/A	N/A	N/A	NI	−	+	NI
			Rib	Me	N/A	N/A	N/A	NI	−	+	NI
9	75	M	Right cervical LN	Me	N/A	N/A	N/A	NI	−	−	−
10	59	M	Left lung	Me	N/A	N/A	N/A	+	−	+	−
			Right adrenal	Me	N/A	N/A	N/A	+	+	+	+
11	44	M	Right cervical LN	Me	N/A	N/A	N/A	+	+	−	+
			Right parotid	Me	N/A	N/A	N/A	+	+	−	+
12	61	M	Right chest wall	Me	N/A	N/A	N/A	+	+	NI	−
13	35	M	Left axillary LN	Me	N/A	N/A	N/A	NI	+	+	NI
14	47	M	Back	R	N/A	N/A	N/A	−	−	+	+
			Right lung	Me	N/A	N/A	N/A	−	−	+	+
15	50	M	Right adrenal	Me	N/A	N/A	N/A	−	+	−	NI
16	43	M	Right post-auricular LN	Me	N/A	N/A	N/A	+	NI	−	−
			Mid-scapula	Me	N/A	N/A	N/A	+	NI	−	−
17	58	M	Left axillary LN	Me	N/A	N/A	N/A	NI	+	+	NI
18	73	F	Right adrenal	Me	N/A	N/A	N/A	NI	−	−	−
			Lymph node NOS	Me	N/A	N/A	N/A	NI	−	−	−
19	75	M	Right cervical LN	Me	N/A	N/A	N/A	NI	−	NI	NI
20	39	M	Right lung	Me	N/A	N/A	N/A	+	NI	+	+

F, female; M, male; P, primary; R, recurrent; Me, metastatic; LN, lymph node; NOS, not otherwise specified; ND, not done; N/A, not applicable; −, no loss of heterozygosity identified; +, loss of heterozygosity identified; NI, sample not informative.

[a] Patient treated with immune therapy prior to excision of primary melanoma, and the specimen was partially submitted for cell culture prior to pathologic evaluation. Accurate pathologic staging thus could not be performed.

[b] Tumor involved the deep excision margin and no subcutaneous tissue was seen. The Breslow depth, Clark's level and TNM stage may thus be greater than reported.

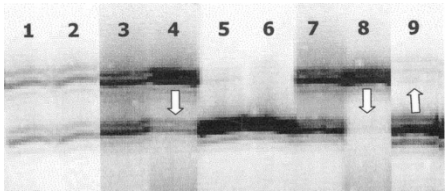

FIGURE 1. MXI1.3NT genotyping of melanomas. **Lanes 1** to **4** represent Patient 1, who is informative for the microsatellite marker, with the specimen from nonlesional dermis showing two bands of similar intensity (**Lane 1).** Both the primary (**Lane 2)** and recurrent (**Lane 3)** melanomas of the left leg show no LOH. The metastasis to the groin lymph node (**Lane 4)** shows LOH with increased intensity of the upper band and a corresponding decrease of the lower (**arrow). Lanes 5** to **6** represent Patient 4, who is noninformative for the gene locus, with only one band present in the normal sample. **Lanes 7** to **9** depict Patient 11 with a primary melanoma of the right cheek (not shown), with informative normal tissue (**Lane 7)** and metastases to the parotid gland (**Lane 8)** and neck lymph node (**Lane 9).** Mutations involved different allelic bands, confirming that each metastasis is derived from genetically distinct clonal populations.

in the English literature to determine its possible involvement in melanoma tumorigenesis. Herbst *et al.* (13) concluded that the low frequency of LOH (7 to 13%) at intragenic markers for *MXI1* in conventional melanomas most likely represented random genetic rather than pathogenic events. Conversely, Rao *et al.* (11) found *MXI1* LOH to be relatively frequent in desmoplastic melanomas, seen in 5 of 11 informative cases.

Our study, unlike that of Herbst *et al.* (13), showed frequent LOH at intragenic and flanking loci of the *MXI1* gene, with aberrations seen in 54% (15 of 28) of the informative cases of primary, recurrent, and metastatic melanomas. This is similar to the 45% LOH that was seen in the desmoplastic melanomas studied by Rao *et al.* (11), although all of our cases were of the conventional type. When the primary melanomas were considered separately, only two of six informative cases (33%) demonstrated LOH. Conversely, 13 of 22 (59%) informative metastatic/recurrent lesions showed genetic loss. Although the number of primary lesions studied is small, this seems to suggest that genetic imbalance involving the *MXI1* gene is a late event in tumorigenesis, associated with metastatic potential and spread. Further anecdotal support for this hypothesis is provided by Case 1, in which no LOH was detected in the primary or recurrent melanoma but there was acquisition of LOH in the groin metastasis at both the intragenic and flanking loci of the *MXI1* gene (Fig. 1).

The findings seen in Patients 10 and 11 are remarkable for their demonstration of genetic heter-

ogeneity in separate metastases from the same primary. In Patient 10, the adrenal and lung metastases were sampled within 1 week of each other, yet the adrenal tumor showed genetic loss in microsatellites for *MXI1* and *MTS1* that the lung lesion did not. The synchronous nature of the metastases begs against genetic progression as is seen in Patient 1 but suggests that two distinct clones developed at the same time in the primary tumor and that both acquired metastatic potential. This is further and more convincingly supported by the findings in Patient 11, in which distinct allelic bands were lost in the *MXI1* gene in the lymph node and parotid gland metastases (Fig. 1). These findings suggest that the molecular evolution of melanoma is not the result of a uniform, linear progression but involves multiple genetic alterations occurring in clonal subpopulations, each attaining its own metastatic potential. These initial findings support those of others (6, 7), but further larger studies need to be performed to investigate the genetic diversity present in melanomas, as well as to correlate these with biologic behavior.

The *MTS1* (*CDKN2A*) gene on chromosome 9p21 has been frequently demonstrated to show allelic loss in both familial and sporadic cutaneous melanomas. This is a tumor suppressor gene that encodes the cell cycle inhibitor $p16^{INK4a}$. In this study, LOH for the *MTS1* gene was seen in 16 of 24 informative melanomas (66.67%), similar in frequency to previous reports of 47 to 72% (2–7, 13). Of the five informative primary tumors, four (80%) showed LOH, also in concordance with other investigators who suggest that mutations involving the short arm of chromosome 9 are early events in the evolution of melanoma (5). The *MTS1* analysis was used as a positive control gene in our study and validates our methodology and findings with respect to the much less frequently studied *MXI1* gene. The finding of similar degrees of allelic loss in both suggests that *MXI1* may also play a role in the development of melanoma, as is believed of *MTS1*.

In summary, the frequency and pattern of allelic imbalances seen in *MXI1* suggest that it is a putative tumor suppressor gene on chromosome 10q involved in conventional melanoma progression. Genetic heterogeneity seen in different metastases from the same primary are indicative of the development of clonally diverse populations, each acquiring its own metastatic potential.

REFERENCES

1. Albino AP. Genes involved in melanoma susceptibility and progression. Curr Opin Oncol 1995;7:162–9.
2. Greene MH. The genetics of hereditary melanoma and nevi. Cancer 1999;86:1644–57.
3. Isshiki K, Seng PA, Elder DE, Guerry D, Linnenbach AJ. Chromosome 9 deletion in sporadic and familial melanomas in vivo. Oncogene 1994;9:1649–53.
4. Healy E, Belgaid C, Takata M, Harrison D, Zhu NW, Burd DA, *et al.* Prognostic significance of allelic losses in primary melanoma. Oncogene 1998;16:2213–8.
5. Healy E, Rehman I, Angus P, Rees JL. Loss of heterozygosity in sporadic primary cutaneous melanoma. Genes Chromosomes Cancer 1995;12:152–6.
6. Morita R, Fujimoto A, Hatta N, Takehara K, Takata M. Comparison of genetic profiles between primary melanomas and their metastases reveals genetic alterations and clonal evolution during progression. J Invest Dermatol 1998;111:119–24.
7. Bogdan I, Xin H, Burg G, Boni R. Heterogeneity of allelic deletions within melanoma metastases. Melanoma Res 2001; 11:349–54.
8. Herbst RA, Weiss J, Ehnis A, Cavenee WK, Arden KC. Loss of heterozygosity for 10q22–10qter in malignant melanoma progression. Cancer Res 1994;54:3111–4.
9. Birck A, Ahrenkiel V, Zeuthen J, Hou-Jensen K, Guldberg P. Mutation and allelic loss of *PTEN/MMAC1* gene in primary and metastatic melanoma biopsies. J Invest Dermatol 2000; 114:277–80.
10. Reifenberger J, Wolter M, Bostrom J, Buschges R, Schulte KW, Megahed M, *et al.* Allelic losses on chromosome arm 10q and mutation of the *PTEN* (*MMAC1*) tumour suppressor gene in primary and metastatic malignant melanomas. Virchows Arch 2000;436:487–93.
11. Rao UNM, Bakker A, Swalsky PA, Finkelstein SD. Max interacting protein 1: loss of heterozygosity is frequent in desmoplastic melanoma. Mod Pathol 1999;12:344–50.
12. Albarosa R, DiDonato S, Finocchiaro G. Redefinition of the coding sequence of the MXI1 gene and identification of a polymorphic repeat in the 3'non-coding region that allows the detection of loss of heterozygosity of chromosome 10q25 in glioblastomas. Hum Genet 1995;95:709–11.
13. Herbst R, Podewski EK, Mommert S, Kapp A, Weiss J. *PTEN* and *MXI1* allelic loss on chromosome 10q is rare in melanoma in vivo. Arch Dermatol Res 1999;291:567–9.
14. Kounelis S, Jones MW, Papadaki H, Bakker A, Swalsky PA, Finkelstein SD. Carcinosarcomas (malignant mixed mullerian tumors) of the female genital tract: comparative molecular analysis of epithelial and mesenchymal components. Hum Pathol 1998;29:82–7.
15. Bussaglia E, Pujol RM, Gil MJ, Marti RM, Tuneu A, Febrer MI, *et al. PTEN* mutations in eight Spanish families and one Brazilian family with Cowden syndrome. J Invest Dermatol 2002;4:639–44.