Cutaneous Tumors in Patients with Multiple Endocrine Neoplasia Type 1 Show Allelic Deletion of the *MEN1* Gene

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Multiple endocrine neoplasia type 1 (MEN1), the heritable tendency to develop tumors of the parathyroid, pituitary, and entero-pancreatic endocrine tissues, is the consequence of a germline mutation in the *MEN1* gene. Endocrine tumors in these patients result when the mutant *MEN1* allele is accompanied by loss of the normal *MEN1* allele. Recently it was reported that MEN1 patients also exhibit several cutaneous tumors, including multiple angiofibromas, collagenomas, and lipomas. The purpose of this study was to examine skin lesions from patients with MEN1 for allelic loss of the *MEN1* gene. Skin lesions from five patients with MEN1 were examined using fluorescence *in situ* hybridization. Six angiofibromas, three collagenomas, and one lipoma showed allelic deletion of the *MEN1* gene. Allelic deletion was not observed in a melanocytic nevus or acrochordon from patients with MEN1. It was also not observed in an angiofibroma from a patient with tuberous sclerosis. These results suggest that loss of function of the wild-type *MEN1* gene product plays a role in the development of angiofibromas, collagenomas, and lipomas in patients with MEN1.

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Multiple endocrine neoplasia type 1 (MEN1) is a dominantly inherited tumor syndrome in which patients characteristically develop tumors of parathyroid glands, entero-pancreatic endocrine tissue, and anterior pituitary (Metz *et al*, 1994). The tendency to develop these tumors results from mutations in a tumor suppressor gene. The *MEN1* gene, known for several years to reside on the long arm of chromosome 11 (Larsson *et al*, 1988), was recently cloned (Chandrasekharappa *et al*, 1997). Mutations in this gene have been reported in 47 kindreds (Agarwal *et al*, 1997).

Tumor suppressor genes are so named because their functional absence leads to the formation of tumors. Because there are two copies of each autosomal gene, both alleles must be inactivated for tumors to develop (the Knudson "two-hit" hypothesis) (Knudson, 1985). In the case of inherited cancer syndromes, one allele is inactivated through a germline mutation. A somatic mutation in the second allele then leads to the formation of a tumor. It has been observed that this "second hit" is commonly a large deletion, encompassing the normal gene along with its flanking DNA (Dong *et al*, 1997). Prior to the identification of a gene, the loss of the gene may be suggested by the loss of polymorphic markers flanking a candidate region. In these studies, normal tissue shows two alleles and the tumor shows only one allele, a finding referred to as loss of heterozygosity (LOH). LOH of polymorphic markers flanking the *MEN1* gene was demonstrated for several tumors in MEN1 (Dong *et al*, 1997, and references therein). Once the gene is identified, however, the deletion of the normal allele can be observed directly by fluorescence *in situ* hybridization (FISH). Thus one may examine individual nuclei for hybridization of a fluorescent probe to each allele of the *MEN1* gene. The presence of only one signal indicates the loss of that gene in the cell. This technique has been used to show allelic deletions at the *MEN1* gene locus in gastrinomas and insulinomas (Zhuang *et al*, 1997).

Recently, we found that patients with MEN1 have the tendency to develop cutaneous tumors including multiple facial angiofibromas [previously considered pathognomonic for tuberous sclerosis (TS)] and collagenomas (Darling *et al*, 1997), in addition to the previously noted lipomas (Ballard *et al*, 1964). The occurrence of these skin lesions raised the question of whether they were a consequence of a "second hit," or secondary to other factors such as haploinsufficiency. Here we show that facial angiofibromas, collagenomas, and lipomas show allelic deletion of the *MEN1* gene.

MATERIALS AND METHODS

Patients Five patients with MEN1 were evaluated in this study. Four are familial cases from large MEN1 kindreds and one case (patient 4) is sporadic, suspected to have a *de novo* mutation. Their ages ranged from 16 y (the youngest MEN1 patient with angiofibromas documented to date) to 69 y (Table I). All of these individuals (patients 1–5) had five or more facial angiofibromas, and all but patient 1 had multiple collagenomas and single or multiple cutaneous lipomas. The cutaneous findings of two patients were reported previously [patients 4 and 5 in this study correspond to patients 27 and 31, respectively, in Darling *et al* (1997)]. The diagnosis of MEN1 was as previously defined (Darling *et al*, 1997). In addition, all five individuals have known mutations in the *MEN1* gene (**Table I**) (Chandrasekharappa *et al*, 1997; Agarwal *et al*, 1997). Two control patients without MEN1 were also examined, control 1 with TS and control 2 with sporadic primary hyperparathyroidism.

Fluorescence *in situ* hybridization Touch preparations of fresh tissue were made at the time of biopsy. Prior to the touch preparation, tissue specimens were blotted of blood, bisected as needed to expose the tumor, and care was

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Abbreviations: FISH, fluorescence *in situ* hybridization; LOH, loss of heterozygosity; MEN1, multiple endocrine neoplasia type 1; TS, tuberous sclerosis.

Table I. Patients with MEN1 show allelic deletion of the *MEN1* gene in angiofibromas, collagenomas, and lipomas

			Skin lesions with allelic deletion by FISH		Skin lesions without allelic deletion by FISH	
#/age/sex	Disease	MEN1 germline mutation[a]	Histologic diagnosis	% Cells with one signal for MEN1 probe (total # cells scored)	Histologic diagnosis	% Cells with one signal for MEN1 probe (total # cells scored)
Patients						
1/16/M	MEN1	R460X	Angiofibroma	63.3 (120)		
2/42/F	MEN1	Y323X	Angiofibroma	48.0 (100)		
			Angiofibroma	52.4 (82)		
3/46/F	MEN1	713delG	Collagenoma	65.3 (95)	Melanocytic nevus	3.3 (91)
			Collagenoma	68.5 (73)		
4/56/F	MEN1	Q260X	Angiofibroma	63.5 (63)[b]		
			Angiofibroma			
5/69/M	MEN1	W436R	Angiofibroma	59.5 (84)		
			Collagenoma	62.5 (48)	Acrochordon	7.1 (84)
			Lipoma	54.5 (66)		
Controls						
1/38/F	Tuberous sclerosis	NA[c]			Angiofibroma	4.4 (91)
2/54/F	Sporadic primary hyperparathyroidism (not MEN1)	NA			Melanocytic nevus	7.1 (85)
					Melanocytic nevus	8.7 (50)
					Melanocytic nevus	2.3 (87)

[a]Chandrasekharappa *et al*, 1997; Agarwal *et al*, 1997.
[b]Because of the low (yet comparable) numbers of cells present, the data for these two angiofibromas are combined.
[c]NA, not applicable.

taken to touch mostly tumor to the slide (dermis for angiofibromas and collagenomas, and fat for lipoma). The tissue was then placed in formalin and processed for light microscopy. Touch preparations were used for FISH analysis as described previously (Pinkel *et al*, 1986; Ried *et al*, 1993). In brief, a cosmid clone c10B11 (size 40 kb) containing the *MEN1* gene was used as a red-signal probe (Guru *et al*, 1997). An alpha-satellite centromeric marker for chromosome 11 was used as a green-signal control. Hybridization signals were scored using a Zeiss (Thornwood, NY) Axiophot epifluorescence microscope and two-color images were captured on a Photometrics CCD camera (Photometrics, Tucson, AZ) using IP Lab image software (Signal Analytics, Vienna, VA). In normal cells, less than 11% of nuclei show only one signal, in part due to overlapping of two signals (Anastasi *et al*, 1990). The presence of more than 30% of cells with only one *MEN1* signal was interpreted as allelic deletion (Zhuang *et al*, 1997).

RESULTS AND DISCUSSION

Angiofibromas in patients with MEN1 show allelic deletion of the MEN1 gene Six facial angiofibromas from five patients with MEN1 were analyzed using FISH. All angiofibromas showed allelic deletion of the *MEN1* gene (**Table I**, **Fig 1**). The percentage of cells with only one signal per nucleus for the *MEN1* probe ranged from 48 to 64%. In some cases, there was also loss of the centromeric marker, suggesting loss of the entire chromosome 11. For controls, two of the patients with MEN1 had FISH analysis of benign skin lesions not associated with MEN1 disease. A melanocytic nevus and an acrochordon did not exhibit allelic deletion of the *MEN1* gene, showing *two* signals per nucleus for the *MEN1* probe in 97 and 93% of cells, respectively. Thus, it appears that allelic deletion of *MEN1* plays a role in the development of angiofibromas in patients with MEN1, but not in other skin lesions that are common to the general population. It should be noted that we have not formally proven that the observed deletion involved the wild-type *MEN1* allele. It is remotely possible that the deletion eliminated the mutant copy of the *MEN1* gene or even a different tumor suppressor gene adjacent to the *MEN1* gene. Very different methods (e.g., allele specific polymerase chain reaction on single cells) would be required to exclude these unlikely mechanisms.

Angiofibromas are also seen in TS. Only 4% of the cells from a TS angiofibroma showed a single signal for the *MEN1* probe (**Table I**). Presumably, this lesion instead possessed deletions in one of the TS genes, as reported previously for cutaneous fibromas from patients with TSC2 (Sepp *et al*, 1996). These data indicate that these identically appearing lesions can arise as a consequence of the loss of distinct tumor suppressor genes involved in entirely different syndromes.

Collagenomas and a lipoma in patients with MEN1 show allelic deletion of the MEN1 gene Three collagenomas from two patients with MEN1 were evaluated for allelic deletion. The percentage of cells with one signal for the *MEN1* probe ranged from 63 to 68% in touch preparations of these tumors (**Table I**, **Fig 1**), indicating that loss of *MEN1* gene function also plays a role in the development of collagenomas. Lastly, a lipoma from one patient with MEN1 showed allelic loss of the *MEN1* gene (54%) (**Table I**). This confirms earlier studies of lipomas in patients with MEN1, which demonstrated LOH at 11q13 in two lipomas, one of which was a large visceral lipoma (Morelli *et al*, 1995; Dong *et al*, 1997).

Melanocytic nevi in a patient with sporadic primary hyperparathyroidism do not show allelic deletion of the *MEN1* gene A patient with sporadic primary hyperparathyroidism had multiple dermal melanocytic nevi (control 2). Touch preps of three of these nevi were analyzed for allelic deletion of the *MEN1* gene, providing additional controls for hybridization efficiency in normal cells. In 91–98% of the cells, two signals for the *MEN1* probe were evident. In summary, control tissues showed one signal per nucleus in 5.5% $\pm$ 2.5% of cells (mean $\pm$ SD, n = 6). The presence of one signal in these nuclei is unlikely to represent the spontaneous deletion of one *MEN1* allele and probably represents insufficient hybridization. A similar low percentage of normal cells showing single-signal hybridization was obtained in a previous FISH study (Anastasi *et al*, 1990). The data for control tissues in this study were used to establish an upper limit of normal, using two standard deviations above the mean as a boundary. Thus the observation of less than 11% of nuclei with a single signal for the *MEN1* probe is within the range of normal. For comparison, no fewer than 48% of cells showed a single signal for the *MEN1* probe in the tissues with allelic deletion.

Implications of allelic deletion in cutaneous tumors These findings suggest that angiofibromas, collagenomas, and lipomas in patients with MEN1 are benign neoplasms of a mono- or oligoclonal nature. Accordingly, a cell with a germline mutation on one *MEN1* allele loses the normal *MEN1* allele, leading to abnormal proliferation of this cell. It is not yet clear which cell population is involved in this genetic alteration in angiofibromas, because angiofibromas are hamartomas showing increased numbers of several cell types (Benjamin, 1996). This cell heterogeneity contributes to the difficulty in demonstrating a "second hit" in these lesions. A prior study of three angiofibromas in patients with MEN1 was unable to demonstrate LOH

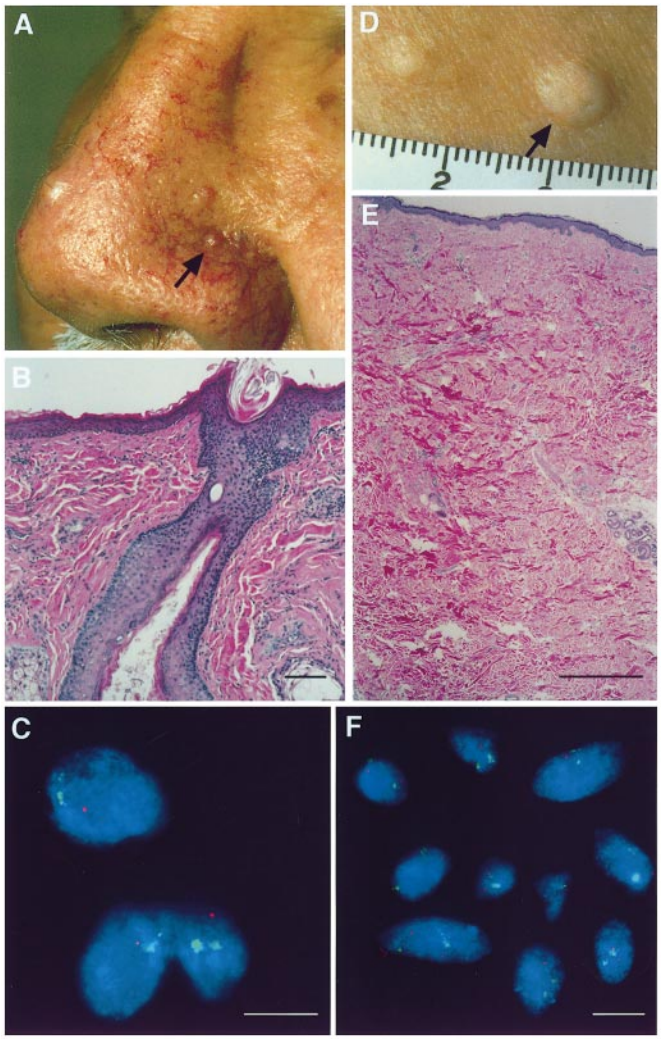

Figure 1. Cutaneous angiofibromas and collagenomas in patients with MEN1 show allelic deletion of the *MEN1* gene. (*A*) Discrete papules are evident on the left of the nose of patient 5, including the lesion chosen for biopsy as indicated by the *arrow*. (*B*) Light microscopy of this lesion shows concentric layers of collagen around vessels and a hair follicle, as seen in angiofibromas. *Scale bar*, 100 μm. (*C*) FISH analysis of a touch preparation of this angiofibroma shows allelic deletion of the *MEN1* gene (red signal) in three nuclei, but the presence of both alleles for the centromeric marker (in green). *Scale bar*, 5 μm. (*D*) On the right shoulder of patient 5 are two dome-shaped papules, including the lesion biopsied as indicated by the *arrow*. (*E*) Light microscopy of this lesion revealed a widened dermis composed of thickened collagen in a haphazard array characteristic of collagenoma. *Scale bar*, 1.0 mm. (*F*) FISH analysis of a touch preparation of this collagenoma demonstrated allelic deletion of the *MEN1* gene (red signal) in six of 10 nuclei. *Scale bar*, 5 μm.

at 11q13 (Dong *et al*, 1997). The ability to show allelic deletion in this study illustrates the utility of FISH in evaluating the genetic makeup of individual tumor cells interspersed amongst other cellular constituents. Further studies are in progress to determine which cell type shows allelic deletion.

Patients with MEN1 can exhibit combinations of multiple angiofibromas, collagenomas, and lipomas. By FISH analysis we have demonstrated that two separate angiofibromas in one patient can show allelic deletion of the *MEN1* gene, and that different skin lesions in one patient can show allelic deletion. Allelic deletion in each lesion presumably arises by independent "second hits," as is the case for the multiple endocrine tumors in patients with MEN1 (Lubensky *et al*, 1996). Furthermore, the second hit is a random occurrence, suggesting that skin lesions should be randomly distributed. This appears to be the case for lipomas in patients with MEN1, and also for neurofibromas in patients with neurofibromatosis, an inherited tumor syndrome in which neurofibromas show LOH for the NF1 gene (Colman *et al*, 1995). Angiofibromas, in both patients with MEN1 and patients with TS, however, predominate on the central face. This localized distribution suggests that additional factors besides allelic loss control whether an angiofibroma may develop. Exposure to ultraviolet light is one possible factor for these lesions on the central face, but ultraviolet light is not characteristically associated with large deletions. It seems more likely that the skin of the central face is sufficiently different from other body sites to facilitate the formation of angiofibromas more easily. This is analogous to the problem of why tumor suppressor genes, widely expressed in different tissues of the body, when inactivated, promote tumor formation in specific organs and not others. With angiofibromas, it is apparent that this specificity extends even to a location within an organ.

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