Perivascular Cells Harboring Multiple Endocrine Neoplasia Type 1 Alterations Are Neoplastic Cells in Angiofibromas

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Abstract

Although neoplasia is caused by clonal proliferation of cells, the resulting tumors are frequently heterogeneous, being composed of both neoplastic and reactive cells. Therefore, identification of tumors as neoplastic processes is frequently obscured. We studied cutaneous angiofibroma, which is a tumor of unknown etiology. Combined analysis using immunohistochemistry, selective tissue microdissection, fluorescence in situ hybridization, sequencing analysis, and deletion analysis of the multiple endocrine neoplasia type 1 locus succeeded in the identification of a population of genetically altered, neoplastic cells in these tumors. This approach may be valuable in the future in identifying the etiology of other tumors of unknown etiology.

Introduction

Neoplasia of tissue is caused by clonal proliferation of cells, resulting in benign or malignant tumors. In many tumors, particularly malignant ones, the proliferating cell population is readily identifiable as an invasive mass that is composed primarily of a single type of tumor cell. The tumor is identified and classified according to the histological and architectural differentiation of the proliferating cell. For example, most colon cancers exhibit characteristic features, such as glandular architecture, epithelial lining, and intracellular and intraglandular mucin, consistent with the differentiation of normal colo-rectal epithelium (1).

Slower-growing benign tumor cells, however, tend to preserve the architectural background. The background stromal tissue may proliferate as well, in response to either physical irritation or factors secreted by the neoplastic cells, and the resulting tumors are frequently composed of different types of cells. In this situation, it may be difficult to elucidate the truly neoplastic component on morphological grounds alone.

Angiofibromas are small tumors of the skin that are composed of a variety of cellular elements, including fibroblasts, neuromuscular cells, dermal dendrocytes, and dilated vessels (2), intermixed with deposits of dermal collagen. Due to the complex histological architecture of angiofibromas, their etiology is currently considered to be hamartomatous rather than neoplastic (2).

Angiofibromas are frequently found in patients with MEN1 (3). Mutation and deletion analyses of MEN1-associated neuroendocrine tumors revealed two genetic hits, germ-line mutation of the MEN1 tumor suppressor gene combined with allelic deletion of the opposite wild-type allele, as an essential pathogenetic mechanism (4–9).

Contrast to the rather monotonous histological pattern of neuroendocrine tumors, however, angiofibromas display a variety of cellular elements, many of which may be reactive rather than neoplastic. In this study, we applied a combined approach of morphological, FISH, and genetic deletion analysis, which succeeded not only in detecting specific genetic changes in angiofibromas but also in localizing the neoplastic component.

Materials and Methods

Tumors. Cutaneous angiofibromas from five patients with MEN1 were retrieved from the files of the Laboratory of Pathology, National Cancer Institute. Multiple tumors were removed from one patient, and touch preparations were prepared from two of them for FISH analysis.

Microdissection. Unstained 6-μm sections on glass slides were deparaf-finized with xylene, rinsed in ethanol from 100 to 80%, briefly stained with H&E, and rinsed in 10% glycerol in 10 mM Tris (pH 8)-1 mM EDTA buffer. A slightly modified microdissection procedure (10) was performed under direct light microscopic visualization using a 30-gauge needle, as described previously. Tumor cells were procured (Fig. 2) from the following areas: (a) control tissue for analysis of constitutional DNA from epidermis; (b) putative tumor cell complexes located around vascular cells; and (c) mesenchymal cells from angiofibroma without putative tumor cell complexes.

DNA Extraction. Procured cells were immediately resuspended in 5 μl of buffer containing Tris-HCl (pH 8.0), 1.0 mM EDTA (pH 8.0), 1% Tween 20, and 0.1 mg/ml proteinase K and were incubated at 37°C overnight. The mixture was boiled for 5 min to inactivate the proteinase K, and 2 μl of this solution were used for PCR amplification of the DNA.

Primers and PCR Conditions. All cases were examined for LOH with microsatellite markers for the MEN1 locus at chromosome 11q13 (D11S449 and PYGM). The markers were chosen on the basis of ease of amplification and informativeness. Each PCR sample contained 2 μl of template DNA, as described above; 10 pmol of each primer; 20 nmol each of dATP, dCTP, dGTP, and dTTP; 15 mM MgCl2; 0.1 unit of Taq DNA polymerase; 0.05 μl [32P]dCTP (6000 Ci/mmol); and 1 μl of 10× buffer in a total volume of 10 μl. PCR was performed with 35 cycles, each consisting of denaturing at 94°C for 1 min, annealing at 55°C for 1 min with D11S449 and PYGM, and extending at 72°C for 90 s. The final extension was continued for 10 min.

LOH Analysis. Labeled amplified DNA was mixed with an equal volume of formamide loading dye (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol). Samples were then denatured for 5 min at 95°C, loaded onto a gel consisting of 6% acrylamide containing 37% formamide and 20 mM EDTA, transferred to 10× SSC solution, and dehydrated in ethanol series of 70, 80, 90, and 100%. In situ hybridization was performed using cosmid clone c10B11 containing the MEN1 as a probe. The DNA was labeled with digoxigenin-11-dUTP by nick translation (Boehringer Mannheim) and...
ethanol-precipitated in the presence of 50× herring sperm DNA and 50× Cot-1 fraction of human DNA. The DNA pellet was resuspended in Hybrisol solution (50% deionized formamide, 10% dextran sulfate, and 2× SSC) to a final concentration of 25 ng/ml. Slides were denatured in 70% formamide-2× SSC at 72°C for 2 min with the following incubation in cold (22°C) ethanol series of 70, 80, 90, and 100% for 2 min each and air-dried. Probes were denatured at 78°C for 10 min and then incubated for 30 min at 37°C for preannealing. A total amount of 250 μg of DNA probe was applied on the slide. α-Satellite repetitive DNA, specific for chromosome 11 (Oncor), was denatured separately and mixed with the cosmid probe just prior to hybridization. Overnight hybridization was done in a humidified chamber at 37°C. Posthybridization washes were at 45°C in 50% formamide-2× SSC (5 min, three times each), 1× SSC (5 min, two times each), and 0.1× SSC (5 min, two times each). Detection was performed using avidin-FITC and anti-digoxigenin-rhodamine (40 min at 37°C), followed by washing in 4× SSC-0.1% Tween 20 solution at 45°C (2 min, three times each) and counterstaining with 4′,6-diamidino-2-phenylindole-antifade (0.25 mg/ml).

Hybridization signals were scored using a Zeiss Axiophot epifluorescence microscope and two-color images were captured on a Photometrics charged coupled device camera (Photometrics, Ltd., Tucson, AZ) using IP Lab Image software (Signal Analytics Corporation, Vienna, VA). At least 100 interphases with strong hybridization signals were scored. Presence of >20% cells with one MEN1 signal was interpreted as an allelic loss. Normal control (normal tissue) showed 3% of cells with one MEN1 signal.

Results

We used the surgical pathology material of five patients with MEN1 and cutaneous angiofibromas (3). In four of the patients, germ-line MEN1 mutations were identified. The tumors measured between 0.3 and 1.0 cm in size and were located at the nose, upper lip, earlobe, chin, shoulder, arm, or groin. From one patient (patient 4), multiple tumors were removed. For initial genetic analysis, angiofibroma tissue was procured by dissecting the dermal angiofibromatous area from H&E-stained slides. Tissue from epidermis and/or adnexal structures adjacent to the angiofibroma nodule was used as normal control tissue. PCR-based LOH analysis of the angiofibromatous tissue, however, consistently failed to reveal LOH. Failure to detect LOH, however, can be interpreted in two different ways:

(a) Angiofibromas consist of cells with constitutive genotype; accordingly, tumor cells and normal tissue from the same patient will yield identical results upon genetic testing. This interpretation would be consistent with the current concept, according to which angiofibromas are not composed of truly neoplastic tissue but rather represent masses of mature, disorganized cells, so-called “hamartomas” (2, 10, 11).

(b) Angiofibromas are composed of truly neoplastic cells, admixed with abundant coproliferating, mature, reactive cells. Consequently,
detection of LOH assays would be obscured by the presence of cells with constitutive genotype.

Two additional studies supported the latter hypothesis. (a) We performed touch preparations from fresh angiofibroma tissue of four MEN1 patients and analyzed the cells using FISH (12). The results showed 48–64% cells with allelic deletion and complementary numbers of cells without allelic deletion (Fig. 1, a and b) and, therefore, provided evidence for a subset of cells to be affected by a “second genetic hit” of MEN1. (b) We performed mutation analysis of 20 angiofibromas from patients without evidence of hereditary disease (13). Although we failed to detect allelic deletion of the MEN1 locus in these sporadic tumors, we identified missense MEN1 mutations in two cases. Again, the results suggested that the analyzed samples consisted of both neoplastic and reactive cells because both a mutation nucleotide and the wild-type nucleotide were detected (Fig. 1, c and d). We concluded from FISH results of MEN1-associated angiofibromas and mutation analysis of sporadic angiofibromas that these tumors contain neoplastic cells that are intermixed with a substantial proportion of nonneoplastic reactive cells. Therefore, detection of allelic deletion by PCR based LOH analysis would require selective procurement and analysis of the neoplastic cell compartment.

Searching for specific cell populations that could potentially represent genetically altered neoplastic cells, we performed immunohistochemical studies of the five cases using consecutive serial sections. In view of the neuroendocrine phenotype of classic MEN1-associated tumors, we applied markers for synaptophysin, chromogranin, and neuron-specific enolase, which, however, failed to detect any immunoreactive cells. Immunostaining with antivimentin, a relatively non-specific marker for a variety of mesenchymal cells, revealed conspicuous clustering of mesenchymal cells around small vessels in all five cases. Cytologically, these cells resembled fibrous or histiocytic dermal cells (Fig. 2). The vimentin-positive cell clusters appeared identical to those described previously as “partial” or complete “perithelial” or “histiocytic” coats or “hamartial germs” (11). The number of the perivascular cell clusters varied markedly between the cases.

Guided by the hypothesis that the clusters of perivascular cells...
Fig. 4. LOH analysis of microdissected cell clusters of eight angiofibromas from four patients with MEN1 with different markers for the MEN1 gene locus showing LOH in perivascular cell clusters. Arrowsheads, locations of both alleles. a, perivascular cell cluster (Lanes 2 and 3) removed and analyzed with PYGM from angiofibroma of patient 1 showing loss of the lower allele; normal control tissue, procured from epidermis and adnexal tissue (Lanes 1 and 4), shows both alleles. Genetic analysis of multiple other neuroendocrine tumors from this patient consistently showed loss of the same allele (data not shown); b, perivascular cell cluster (Lane 1) removed and analyzed with PYGM from angiofibroma of earlobe of patient 2 showing loss of the lower allele; normal control tissue, procured from epidermis and adnexal tissue (Lanes 2 and 3), shows both alleles. c, perivascular cell cluster (Lane 1) removed and analyzed with PYGM from nasal angiofibroma of patient 3 showing loss of the upper allele; normal control tissue, procured from epidermis and adnexal tissue (Lanes 2, 3, and 4), shows both alleles. d, three different clusters removed and analyzed with D11S449 from angiofibromas of the upper lip (Lanes 3 and 4) and chin (Lane 6) of patient 4; normal control tissue was procured from epidermis and adnexal structures (Lanes 1, 2, and 5). The tumor areas show loss of the lower allele. e, multiple different perivascular cell clusters (Lanes 2, 4, and 7–16) taken from four different angiofibromas (patient 4). Some tumor samples show no LOH (Lane 2) or allelic imbalance (Lane 4), suggesting “contamination” with reactive cells with constitutive genotype; others show unequivocal LOH (Lanes 7, 8, 11, 12, 14, and 16). No amplification product was obtained from four samples (Lanes 9, 10, 13, and 15). Normal tissue (Lanes 1, 3, 5, and 6) was procured from epidermis and adnexal structures from different slides. Whenever LOH is observed in perivascular tumor cell clusters in this patient, there is loss of the lower allele.

Discussed tumor syndromes serve as valuable models for detailed genetic study. Tumors occur in different organs, and frequently, they occur in multiplicity within the same organ. Each individual tumor appears to arise independently, and knockout of the wild-type tumor suppressor allele is hypothesized to represent an early or even initiating event (15). Furthermore, extensive studies of tumors in various hereditary tumor syndromes, including MEN1, von Hippel-Lindau disease, and others, have characterized the second hit as allelic deletion. Therefore, the tumor cells in these hereditary syndromes are genetically “marked” by a deletion event that can be sensitively detected whenever the tumor cells are selectively procured. In contrast, procurement of nonneoplastic cells (or of tissue probes consisting of predominantly nonneoplastic cells) will demonstrate presence of both tumor suppressor gene alleles.
Here, we applied selective tumor cell analysis to cutaneous angiofibromas, which frequently occur in association with MEN1. Angiofibromas are benign tumors that are predominantly composed of mesenchymal cell complexes and vessels. On the basis of the bland morphological features, these tumors have been considered to represent reactive/hamartomatous masses rather than true neoplasia.

Two lines of evidence, however, suggested that angiofibromas are not exclusively composed of reactive cells. The first evidence was provided by the observation of MEN1 deletion with cosmid clone c10B11 containing MEN1 in tumor nuclei that had been prepared by touching angiofibroma tissue onto glass slides (12). The second evidence was derived from SSCP and sequencing analysis of sporadic angiofibromas, which revealed bands representing both constitutive and aberrant genotypes (13). The applications of both FISH and/or SSCP/sequencing analysis, however, are limited because they do not allow to study cells in their original architectural context; therefore, morphology and location of the genetically altered cells remained unknown.

In patient 1, whose cutaneous lesions were analyzed in this study, we were consistently able to demonstrate MEN1 deletions in other MEN1-associated tumors. Consistently, it was the same allele that was lost in each individual neoplasm. However, initial analysis of this and other MEN1 patients’ angiofibromas failed to reveal MEN1 deletion. In retrospect, the absence of MEN1 deletion was caused by an abundance of tissue cells with normal constitutive genotype. In contrast, after selective procurement of perivascular cell complexes, previously called hamartial germs (11), we were consistently able to demonstrate loss of one MEN1 allele.

Although our study provides evidence for the presence and the location of neoplastic cells in angiofibromas, the histogenesis of these cells remains unclear. Recent experiments support the concept that MEN1-associated tumors may originate from pluripotent cells that may differentiate along different pathways, including mesenchymal and epithelial lineage (16). The rich vascularization of angiofibromas and noncutaneous, MEN1-associated neuroendocrine tumors may indicate angiogenic properties of neoplastic cells with MEN1 deletion.

In conclusion, we support previous evidence that MEN1-associated angiofibromas arise as part of the spectrum of MEN1 disease that has so far been predominantly characterized by development neuroendocrine tumors. MEN1-associated angiofibromas represent true neoplastic processes. The neoplastic cells are concentrated in perivascular location and reveal mesenchymal immunophenotype. Further studies will have to closer characterize the neoplastic cell in other MEN1-associated tumors.

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References

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