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Evidence for the Presence of the Second Allele of the Neurofibromatosis Type 1 Gene in Melanocytes Derived from Café au Lait Macules of NF1 Patients

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Neurofibromatosis type 1 (NF1) is an autosomal dominantly inherited disorder caused by mutations in the NF1 gene on 17q11.2. Melanocytes cultured from café au lait macules (CALM) of patients with NF1 were analysed for loss of heterozygosity (LOH) at the NF1 locus using a 3′-flanking and four intragenic markers. None of the informative samples showed LOH. In addition, the X-inactivation pattern of melanocytes from CALM (n = 4) and from the unaffected skin of the patients (n = 3) suggests a monoclonal origin of the cells isolated from skin biopsies up to 2 cm² in size.

Neurofibromatosis type 1, one of the most common genetic disorders in humans, is characterized by multiple neurofibromas and anomalies of pigmentation, e.g., café au lait macules (CALM). NF1 patients have also an increased incidence of certain malignant tumors. Additionally a variety of further complications have been described, albeit the presence and the severity of the symptoms is highly variable (1). NF1 can therefore be described as an autosomal dominantly inherited disease with pleiotropic manifestations and a high degree of variable expressivity. Malignancies in NF1 are believed to follow the hypothesis of the two-hit, stepwise inactivation of a tumor suppressor gene, in which one allele is constitutionally inactivated while the other allele is subsequently inactivated at the somatic level (2, 3, 4, 5). Recently the same mechanism has been described for some beningn neurofibromas of NF1 patients (6, 7). Hence, the question arises, whether the other benign lesions of NF1, especially the anomalies of pigmentation, also arise according to the two-hit hypothesis. To address this question for the pigmentation anomalies of NF1 patients, we searched for the postulated second-hit at the NF1 locus in CALM-melanocytes from NF1 patients. Most of the somatic mutations that inactivate or delete the wildtype allele of a tumor suppressor gene in constitutionally heterozygous cells can be detected as "loss of heterozygosity" (LOH) at informative intragenic or closely flanking markers.

Melanocytes from human skin can be raised and maintained as pure cultures (8), enabling the search for LOH in melanocytes that originate from the café au lait macules of the patients.

METHODS

NF1 patients were diagnosed according to the criteria of the NIH Consensus Statement (9). Melanocytes were isolated from skin biopsies, raised and maintained in culture as described previously (10). Isolation of melanin-free DNA was carried out with the DNA-isolation-Kit from Qiagen with minor modifications from the protocoll supplied by the manufacturer. All PCR primers used in this study were described earlier (11, 12, 13, 14, 15, 16). In each case one of the PCR primers was labelled with Cy5 for subsequent product analysis after separation with 6% polyacrylamide gels on the Pharmacia ALF express sequencer. Analyses of the product patterns were done with the ALF fragment manager as software tool. For the X-inactivation studies aliquots of the genomic DNA samples were digested with Hpa II prior to PCR.

RESULTS AND DISCUSSION

Melanocyte cultures from 11 unrelated NF1 patients were included in the following investigations. LOH studies were carried out by analysis of five different polymorphic markers from the NF1 gene locus (11–15). Four of the markers are located in the NF1 gene (RsaI, Alu, 28.4, 53.0), whereas the fifth marker (Mfd15) maps distal to the gene. All polymorphisms were analysed by PCR-amplification with flanking primers and subsequent acrylamide gel-electrophoresis of the products using the ALF express sequencer. The analyses of the product patterns were carried out with the ALF fragment manager as software tool. In each case, the amplification patterns obtained with

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TABLE 1

Summary of the Results Obtained during the Search for Loss of Heterozygosity in Melanocytes Derived from Café au Lait Macules of NF1 Patients

NF1 patient, origin of melanocyte culture	Polymorphism, location				
	RsaI NF1 exon 5	AluI NF1 intron 27b	28.4 NF1 intron 27b	53.0 NF1 intron 38	Mfd 15 distal from the NF1 gene
NF10, female	○	○	○	◍	○
NF25, male	○	◍	○	◍	◍
NF31, male	○	○	◍	◍	○
NF52, female	◍	◍	◍	◍	○
NF71, male	◍	◍	◍	◍	○
NF92, female	○	○	○	○	○
NF97, male	◍	◍	◍	◍	◍
NF106, male	◍	◍	◍	○	◍
NF128, female	◍	◍	◍	◍	◍
NF137, female	○	◍	○	◍	◍
NF224, male	○	○	◍	○	○

Note. Five polymorphic markers from the NF1 gene locus were included in these experiments.
Legend: ○, not informative, ◍, heterozygous.

DNA from the CALM-melanocytes were compared to the genotype of melanocytes from the normal skin, in some cases with that of blood cells from the patients. An overview of the results is given in Table 1. Both alleles of the NF1 gene were present in melanocytes from CALM in all informative cases (10 of 11). Two patients (NF128 and NF97) were heterozygous for all polymorphic markers under investigation and clearly, both alleles of the NF1 gene were present in the DNA isolated from the cultured CALM-melanocytes in both cases. Further two patients (NF71 and NF52) were informative for all of the intragenic markers allowing also conclusions with regard to the status of most of the NF1 gene in the CALM-cells from these patients. Six of the seven remaining patients were informative at one to three of the markers and heterozygosity could be demonstrated in the CALM-melanocytes also of these patients. Only one patient (NF92) turned out to be homozygous for all the markers applied. These results exclude the loss of the wildtype allele by mechanisms like large deletions, non-disjunction, somatic recombination and gross rearrangements, since the five markers cover a wide region of the gene (from exon no. 5 to a region distal of the gene). Unfortunately there is still no description of a further polymorphic marker for the most 5′ part of the NF1 gene, so that this region is excluded from the screening.

For the benign tumors of NF1 patients Colman et al. (6) demonstrated LOH in 8 of 22 neurofibromas with a panel of polymorphic markers which included the ones that we used in our studies. Unfortunately, the only second hit identified by Sawada and coworkers (7) was a small 4 bp deletion occurring in a subset of cells of the neurofibroma. Däschner et al. (17) found one case with LOH at the NF1 gene among the 38 neurofibromas tested. Neurofibromas are known to consist of a variety of cell types, further complicating the search for the second event in these benign tumors. But this is not comparable with the investigations employing melanocytes derived from CALM, since all our studies were done employing pure cultures of these cells. Nevertheless, we cannot strictly rule out any functional inactivation of the wildtype allele by point mutation or altered methylation pattern of the gene in CALM-melanocytes but as described for other tumor suppressor genes so far, most of the inactivation processes of the wildtype allele occur at the chromosomal level (nondisjunction, somatic recombination) or are based on large deletions (18) and therefore lead to detectable LOH. These events would have been found with the methods applied under the assumption that the melanocytes which colonize a CALM are indeed of homogenous origin. On the other hand, if more than one precursor cell immigrates to the area of the presumptive CALM, producing a mixed population of cells with and without a second event at the NF1 gene locus, we would not be able to find LOH in the cells originating from the CALM.

To examine the clonal origin of the melanocytes from CALM and unaffected skin of NF1 patients, we analyzed the X-inactivation pattern of the melanocytes derived from the normally pigmented skin and CALM of our female NF1 patients. The X-chromosomally located androgen receptor gene carries a highly polymorphic microsatellite repeat in exon no. 1, which is neighboured by two restriction sites for the methylation sensitive enzyme HpaII (16). PCR-amplification with

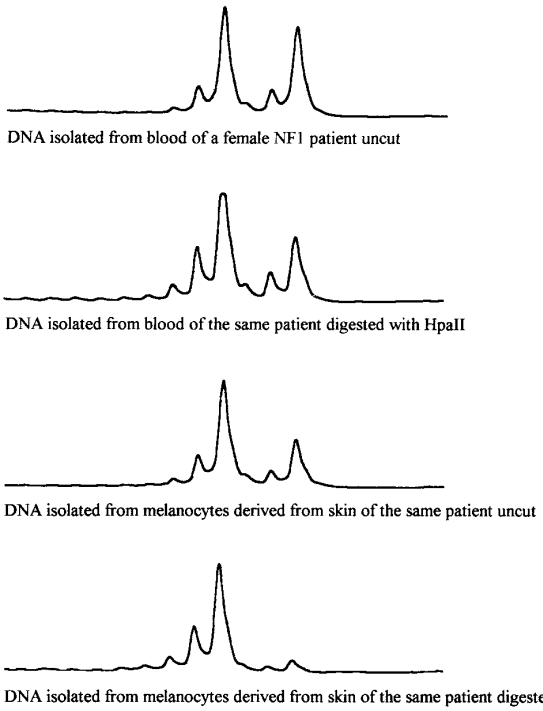

FIG. 1. Curves obtained after electrophoretic separation of Cy5-labelled PCR products generated from the microsatellite repeat in exon 1 of the androgen receptor gene. The separation and detection of the fragments were performed with the ALF express sequencer.

primers flanking the repeat as well as the restriction sites, allows to distinguish between the methylated inactive X-chromosome and the unmethylated active X-chromosome in female cells. PCR amplification after HpaII digestion of the genomic DNA generates PCR-products of both alleles in tissues of polyclonal origin e.g. blood, whereas PCR-amplification employing DNA isolated from cells or tissue of monoclonal origin generates products only from the highly methylated inactive X-chromosome. An example of the results obtained with human melanocyte cultures is shown in Fig. 1. DNA isolated from blood allows the amplification of both alleles from this heterozygous female patient, before as well as after HpaII digestion. PCR-amplification of DNA isolated from melanocyte cultures of the skin of the same patient generates both products before digestion, but after the digest only the smaller allele of the repeat is amplified. Similar results were obtained with all informative melanocyte cultures examined (n = 7; 4 CALM, 3 Skin), irrespective of their origin from the normal skin or a café au lait macule of the female patients. These results suggest, that the area colonized by the progeny of one melanocyte precursor is larger than the excised patch of skin. We were not able to draw conclusions about the whole CALMs since the excised patches were always smaller than the whole CALM of the patients.

Further investigations revealed, that the protein product of the NF1 gene, neurofibromin, could be detected by immuno-precipitation and Western Blot analysis of the precipitates in all melanocyte cultures from CALM tested. Western blotting was carried out with an antibody binding to the carboxy-terminus of neurofibromin. Since some of the patients harbour a constitutional mutation leading to a stop codon at the 5′ end of the coding region of the gene, only protein translated from the wildtype allele is detected by the antibody in these cases (19). These data confirm the immunohistochemical detection of neurofibromin in the melanocytes of the basal layer of CALM epidermis from NF1 patients, reported by Malhotra and Rattner (20).

Since all melanocyte cultures examined showed a clonal X-inactivation pattern and both alleles of the NF1 gene were found in CALM-melanocytes in all informative cases we have to consider one of the following hypotheses for the generation of CALM: 1. Melanocytes are not the primarily affected cells in CALM. 2. Melanocytes are the primarily affected cells but both normal and affected cells are present in a CALM. 3. Cells without neurofibromin do not grow in culture. Melanocyte cultures are selected for cells that retained neurofibromin. 4. Different genetic mechanisms are responsible for the generation of neurofibromas and CALM in NF1. Further investigations will be necessary to reveal the mechanisms leading to CALM formation in NF1 patients.

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