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BRIEF COMMUNICATION

Exclusion of the Nuclear Factor- κ B3 (REL A) Gene as Candidate for the Multiple Endocrine Neoplasia Type 1 (MEN 1) Gene

Multiple endocrine neoplasia type 1 (MEN 1) is inherited as an autosomal dominant disorder, characterized by neoplasia and hyperplasia in specific endocrine organs. The MEN 1 gene, which is most probably a tumor suppressor gene, has been localized to a region of approximately 900 kb on chromosome 11q13. The nuclear factor- κ B (NF- κ B) is a transcription factor with pleiotropic expression, which is involved in the regulation of expression of many cellular genes. The p50/p65 heterodimer is the most abundant form of NF- κ B. The gene encoding the p65 subunit (NF- κ B3/REL A) was recently localized in the 900-kb MEN 1 region and was considered a good candidate gene for MEN 1. The structure and nucleotide sequence of the NF- κ B3 coding region in MEN 1 patients were compared with those of non-MEN 1 subjects, to determine the potential role of this gene in MEN 1 tumorigenesis. Southern blot analysis with constitutional DNA from probands of 14 independent MEN 1 families and DNA from four MEN 1 tumor specimens did not reveal any structural abnormality of the NF- κ B3 gene. Direct sequencing of cDNAs from two affected subjects from 2 different MEN 1 families, as well as nucleotide sequence analysis of exon/intron boundaries in these patients, did not reveal MEN 1-specific point mutations or other small structural aberrations in the NF- κ B3 gene. These results make it very unlikely that the NF- κ B3 gene is the gene responsible for the development of MEN 1. © 1997 Academic Press

Multiple endocrine neoplasia type 1 (MEN 1) is a familial cancer syndrome with an autosomal dominant pattern of inheritance. The disorder is characterized by the combined occurrence of hyperplasia or tumors of the parathyroid glands, the pancreatic islets, and the anterior pituitary gland (1). Using linkage analysis in MEN 1 families and allelotyping

of MEN 1-associated tumors, the hereditary genetic defect had been assigned to chromosome 11q13. Elimination of the wild-type allele at 11q13 loci in MEN 1 tumors suggests inactivation of a tumor suppressor gene in this region as the causative mechanism in MEN 1 tumorigenesis (2).

Loss of heterozygosity studies revealed a smallest region of overlapping deletions in MEN 1-associated tumors, flanked by the loci D11S427 and D11S97 (3). Previous linkage analysis showed meiotic recombinants in MEN 1 families for the locus D11S807 (4). These studies place the MEN 1 locus between the centromeric marker D11S427 and the telomeric marker D11S807, a region of at most 900 kb (3).

Nuclear factor- κ B (NF- κ B) represents a family of closely related homo- and heterodimers, whose members belong to the Rel protein family of transcriptional activators. This family includes p50 (NF- κ B1), p52 (NF- κ B2), p65 (NF- κ B3/RelA), Rel (c-Rel), and RelB (5). All these proteins are homologous to the v-Rel oncoprotein (6). The NF- κ B3 gene had been localized to chromosome 11q11–11q13, near the ROM1 gene (7,8), centromeric to the 900-kb MEN 1 region. However, recently it was shown that the NF- κ B3 gene maps within this MEN 1 region (9). Because of its chromosomal location, the role of its encoded protein in transcription modulation, and its ubiquitous expression pattern (as can be expected for a tumor suppressor gene), the NF- κ B3 gene was considered a good candidate for being the MEN1 gene.

In this study, the structure and protein-encoding sequence of the NF- κ B3 gene in MEN 1 patients were examined to detect possible MEN 1-specific abnormalities. Southern blot analysis of constitutional DNA from affected subjects from 14 MEN 1 families, as well as DNA from four MEN 1-associated tumors,

was performed to screen for large aberrations in the NF- κ B3 gene. Additionally, we screened for point mutations and other small aberrations, by sequencing the entire protein-encoding region of NF- κ B3 cDNA, synthesized on fibroblast RNA and tumor RNA from affected subjects from 2 MEN 1-families.

MATERIALS AND METHODS

DNA Isolation and Southern Blot Analysis

Blood was obtained from MEN 1 patients and non-affected family members from 14 unrelated families. Tumor tissue was obtained from two glucagonomas, one insulinoma, and one pituitary gland adenoma from four individual patients. DNA was isolated from blood and tumor samples according to Miller *et al.* (10). *EcoRI*- and *TaqI*-digested DNA (10 μ g) was size-fractionated in 0.8% agarose gels and blotted onto Nylon⁺ membranes (Hybond, Amersham, UK). Hybridizations were performed using a cosmid (cC21) containing the entire NF- κ B3 gene, as well as 5' and 3' flanking sequences (11). This clone was isolated from a grided flow-sorted chromosome 11-specific cosmid library, kindly provided by Drs. Leharach and Zehetner (Max-Planck Institute, Berlin, Germany). Hybridizations were performed as described previously (12). The membranes were exposed overnight to a Phospho-imager screen (Molecular Dynamics, Sunnyvale, CA), or to X-ray films for 1–7 days.

Nucleotide Sequence Analysis

The entire 1653-nucleotide coding region of NF- κ B3 mRNA was analyzed by sequencing overlapping DNA fragments obtained after RT/PCR (Fig. 1). RNA was isolated from a frozen sample of glucagonoma tissue and from a fibroblast cell culture, using RNA-SR according to the manufacturer's instructions (Biogenesis, Bournemouth, UK). cDNA was synthesized using Superscript reverse transcriptase (BRL, Breda, The Netherlands) and either a mixture of oligo(dT)_{12–18} and random hexamers or NF- κ B3-specific oligonucleotides as primers (the nucleotide sequences and locations of the primers used for RT/PCR and nucleotide sequence analyses are available on request). PCR was performed as described previously (13). The PCR products were analyzed by agarose gel electrophoresis. Specific products were isolated from ultra low melting point agarose gels and used directly for nucleotide sequence analysis using a pUC sequencing kit (Boehringer, Mannheim,

Germany) with [α -³⁵S]dATP as the label. Sequencing products were separated on 6% polyacrylamide gels, which were exposed to X-ray films.

RESULTS AND DISCUSSION

For the Southern blot analysis, *TaqI* and *EcoRI* restriction patterns of the NF- κ B3 gene in blood and tumor DNA from MEN 1 patients were compared to the restriction patterns in DNA from blood from two non-MEN 1 subjects. In the constitutional DNA, only the germ line mutation predisposing to MEN 1 might be detected, whereas the tumor DNA might also reveal a somatic mutation, representing the second hit in Knudson's model for hereditary cancer (14). We could not detect an aberrant restriction pattern for any of the digests in either blood DNA or in tumor DNA (Fig. 2). Therefore, we decided to screen for smaller MEN 1-specific changes, which would result in the production of mutant mRNA, by sequencing the entire protein-encoding region of NF- κ B3 mRNA. For this purpose, we performed RT/PCR on RNA from a MEN 1 glucagonoma (thereby screening for a germ line mutation as well as for somatic mutations) and on RNA from a fibroblast cell line from another MEN 1-patient, thereby screening for the germ line mutation only.

Four partially overlapping DNA fragments were generated with the selected primers, as outlined in Fig. 1. The entire coding region of NF- κ B3 cDNA was sequenced for both MEN 1 tissues. No MEN 1-specific nucleotide sequence variants were detected, when compared with NF- κ B3 cDNA sequences reported previously (15) (EMBL Accession Nos. Z22948-Z22954, inclusive). The intron/exon junctions were sequenced using intron-specific primers after PCR of patients' DNAs. No mutation was found in any case. Since we did not detect any common nucleotide sequence polymorphism in the RT/PCR products, we were unable to prove that both alleles of the NF- κ B3 gene were expressed and analyzed. Theoretically, in the glucagonoma tissue analyzed, one allele of the NF- κ B3 gene might be missing/inactivated due to a tumor-specific somatic mutation (second hit). In that case we have sequenced the allele which should carry the germ line mutation (first hit). However, such a mutation was not detected. In the fibroblast cell culture, both alleles of the NF- κ B3 gene should be present and expressed, unless the germ line mutation in the family of this MEN 1 patient involves deletion/inactivation of this gene. In contrast, for example, to Von Hippel-Lindau

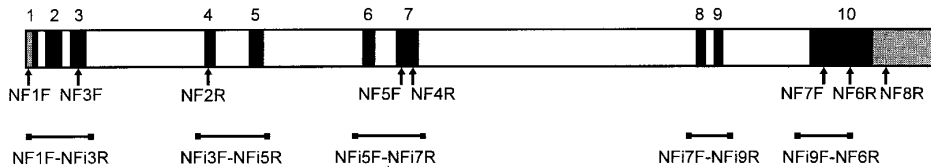


FIG. 1. Schematic representation of the gene encoding NF- κ B3. Exons are numbered 1–10; black boxes indicate protein-encoding regions, shaded boxes indicate untranslated regions. Arrows indicate the location of oligonucleotide primers which were used to synthesize four overlapping RT/PCR products on RNA (NF1F-NF2R, NF3F-NF4R, NF5F-NF6R, NF7F-NF8R). Bars at the bottom indicate PCR products made on genomic DNA, for the analysis of intron/exon boundaries (detailed information on oligonucleotides available on request).

disease (VHL), where approximately 20% of the families have a large germ line deletion in the VHL tumor suppressor gene (16), germ line deletions in the MEN 1 locus have not yet been detected in MEN 1 families, making this possibility unlikely for the two patients we investigated.

In summary, no deletions, insertions, rearrangements, or MEN 1-specific point mutations were detected in NF- κ B3 cDNA in two unrelated MEN 1-patients. Because no alternative transcripts were detected after RT/PCR and subsequent nucleotide sequence analysis, there were no indications for mutations at exon–intron junctions. In addition,

all intron–exon junctions were sequenced on PCR amplicons generated on DNA from these two affected subjects. In conclusion, these results make it highly unlikely that the NF- κ B3 gene is the MEN 1 gene.

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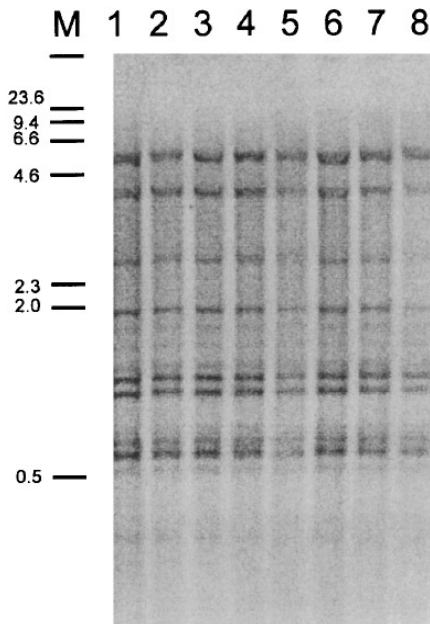


FIG. 2. Southern blot analysis of *Taq*I-digested DNA. Hybridization probe was cosmid C21 which contains the complete NF- κ B3 gene, as well as 5' and 3' flanking sequences. Lanes 1–6, blood from MEN 1-patients from five different families; lane 7, blood from an unrelated non-MEN 1 individual (control); lane 8, DNA from a MEN 1-associated pituitary tumor. M, length marker in kb (*Hind*III digest of bacteriophage λ DNA).

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