A G→A transition creates a branch point sequence and activation of a cryptic exon, resulting in the hereditary disorder neurofibromatosis 2

Annelies De Klein1,4, Peter H.J. Riegman2, Emilia K. Bijlsma5, Anneliek Heldoorn1, Manja Muijtjens1, Michael A. den Bakker2, Cees J.J. Avezaat3 and Ellen C. Zwarthoff2,*

Departments of 1 Cell Biology and Genetics, 2 Pathology, 3 Neurosurgery and 4 Ophthalmology, Erasmus University, PO Box 1738, 3000 DR Rotterdam, The Netherlands and 5 Department of Human Genetics, Academic Medical Centre, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands

Received August 21, 1997; Revised and Accepted December 15, 1997

We describe a G→A transition within intron 5 of the NF2 gene. This mutation creates a consensus splice branch point sequence. To our knowledge this is the first report of a mutation that creates a functional branch point sequence in a human hereditary disorder. The new branch point sequence is located 18 bp upstream of a consensus splice acceptor site. A consensus splice donor site is found 106 bp 3′ of the acceptor site. As a consequence the G→A transition results in an alternatively spliced mRNA containing an additional exon 5a of 106 bp derived from intron sequences. We cloned the mutant cDNA and show that due to an in-frame stop codon the cDNA codes for a truncated NF2 protein. The mutation was observed in three affected members of an NF2 family. In a tumour of one of the family members both alternatively spliced and wild-type mRNA were found, although the wild-type allele of the gene is absent due to an interstitial deletion on chromosome 22. We also show that immuno-precipitations reveal the presence of full-length wild-type NF2 protein in the tumour lysate. These data support the hypothesis that some degree of normal splicing of the mutant precursor RNA is taking place. It is therefore likely that this residual activity of the mutant allele explains the relatively mild phenotype in the family. These data also indicate that complete inactivation of the gene is not required for tumour formation.

INTRODUCTION

Branch point sequences are located 18–40 nt upstream of a splice acceptor site, which is usually the first AG downstream of the branch point sequence. In the branch point consensus sequence YNYURAC (Y = C or T; R = G or A; N = any base) the A at position 6 is critical for formation of a mammalian splice intermediate, the lariat structure. In addition, it has been shown that mutation of this A residue strongly reduces splicing efficiency of the downstream exon (1). In human genetic diseases splicing errors are common; however, most of these alterations are due to a mutation of the splice donor or acceptor site. Only two cases have been described so far in which the splicing error was the result of a mutation of the invariant A residue of the branch point resulting in reduced usage of the downstream exon (2,3). Neurofibromatosis type 2 (NF2) predisposes patients to development of central nervous system tumours such as Schwannomas, meningiomas and ependymomas (4,5). Additional manifestations of the disease include peripheral Schwannomas and cataract. NF2 is an autosomal dominant disorder with a birth incidence of 1:35 000. The NF2 gene, located on chromosome 22q12, consists of 17 exons, of which exons 16 and 17 are alternative splice forms leading to different C-termini of the NF2 protein (6–8). Mutations in the NF2 gene have been found in NF2 family members and in sporadic Schwannomas and meningiomas (9). In addition, mutations were found in malignant mesotheliomas, a tumour that does not develop in NF2 patients (10,11).

Most of the mutations that have so far been described in the NF2 gene predict that gene function is negatively affected. In general, mutations that lead to truncation of the protein are found more frequently in patients with the severe form of the disease. In patients with a mild phenotype missense mutations are more frequent. This suggests that there is a correlation between phenotype and genotype (9,12,13). It has been speculated that the mutations leading to a truncated protein could perhaps function as dominant negative mutations in which the truncated protein interferes with function of the wild-type gene. The reduced activity of the NF2 protein could then trigger some degree of hyperplasia, which would increase the chances of a second hit (i.e. loss of the other allele), after which tumour formation would ensue. Such a scenario would explain the larger number of tumours in severely affected patients. However, this hypothesis remains to be proven. Mutations that affect splice sites are encountered in both forms of NF2 and it has been argued that in
cases with the mild form of NF2 these mutations do not completely prevent normal splicing. However, the effect of the mutation on mRNA and protein has not been investigated.

In the current report we describe a G→A transition which causes NF2 in a Dutch family. The mutation creates a consensus splice branch point sequence in intron 5 of the NF2 gene. Due to the presence of downstream splice acceptor and donor sites this mutation results in incorporation of an additional exon in the NF2 mRNA. We show that the alternative mRNA codes for a truncated protein. In addition, we detected large amounts of wild-type mRNA and protein in tumour tissue. This suggests that the mutation still allows normal splicing of the mutant allele. This finding might explain the relatively mild phenotype in the family and indicate that tumorigenesis does not require complete absence of wild-type NF2 protein.

RESULTS
Detection of a point mutation in the NF2 gene

Earlier we reported that in a meningioma from case 2 (MN121) the NF2 cDNA contained an extra insert of 106 bp (14). The insertion of 106 bp located between exons 5 and 6 suggested a mutation either in the exon 5 splice donor site or in the exon 6 splice acceptor site. In order to find the exact position of the mutation in genomic DNA we have amplified these exons, including the exon/intron borders with exon 5- and 6-specific primers (15). No altered migration pattern was observed on agarose gels or by SCCA analysis (not shown) nor did the sequence of the 106 bp insertion correspond to the reported exon border sequences. These results indicate that mutations in the exon 5 splice donor or exon 6 splice acceptor sites were not the cause of the insertion.

To determine the origin of the extra 106 bp at the exon 5 and 6 border in MN121 cDNA we cloned a 1.0 kb PCR fragment encompassing exons 5 and 6 and the intron 5 sequences of the NF2 gene from case 2. To locate the 106 nt cDNA insertion in the intronic sequences we used the restriction enzyme BpmI, which has one recognition site in the 106 nt insertion. BpmI also cuts once in the 1.0 kb genomic exon 5–6 fragment. By determining the sizes of the restriction fragments we were able to locate the 106 nt insertion 249 bp downstream of exon 5 (Fig. 1A). PCR using primers from exon 5 or exon 6 in combination with primers derived from the 106 nt sequence confirmed the position of the 106 bp sequence. These primers were also used for DNA sequencing and the sequence of a 430 bp genomic region of MN121 DNA was determined and compared with normal DNA (Fig. 1). It appears that in both control DNA and in DNA from tumour MN121 the 106 nt insertion is present. However, in MN121 there is a G→A point mutation at position 301 (the start of exon 5 is designated 1), 18 bp upstream of the 106 bp sequence. This is the only difference we could detect in MN121 tumour DNA when compared with the control. The point mutation results in the creation of a putative branch point site just in front of a putative splice acceptor site (Fig. 1). The splice acceptor site precedes the genomic copy of the 106 bp insertion. At the 3′-end the 106 bp genomic sequence is flanked by a putative splice donor site. We therefore conclude that the point mutation creates a new splice branch point sequence which, in combination with already existing consensus splice acceptor and donor sites, results in formation of an extra exon 5a in the NF2 gene.

Effect of the mutation on the NF2 protein

To analyse the NF2 protein encoded by cDNA carrying the insertion we generated an expression plasmid (pR121) containing a cDNA derived from the mutant NF2 transcript from meningioma MN121. This mutant cDNA encodes a putative protein of 23.5 kDa consisting of 172 amino acids of the normal NF2 protein and 31 amino acids derived from exon 5a. A nonsense codon is present at position 412 in exon 5a (see Fig. 1). When clone pR121 was used for in vitro transcription/translation a truncated protein with the expected molecular weight of ∼25 kDa was found (Fig. 2A). In addition, a western blot of a lysate of COS cells transiently transfected with the pR121 construct also showed a protein of ∼25 kDa (results not shown).

To see whether the truncated protein could be detected in MN121 we used a frozen sample of this tumour for immuno-
Figure 2. Analysis of the NF2 protein. (A) In vitro transcription/translation of wild-type and mutant (R121) cDNA. The positions of the wild-type (wt) NF2 protein, which migrates at ~70 kDa, and the mutant (mt) 23 kDa protein are indicated. (B) Immunoprecipitation of the NF2 protein from a lysate of meningioma MN121. The wild-type NF2 protein produced by transfected COS cells is used as a control for the position of the full-length NF2 protein. The bands below the full-length NF2 band in MN121 are from the precipitating antibody. The identity of the slower migrating band, which is sometimes observed in these analyses (den Bakker, unpublished results), is not known at present. The 23 kDa mutant NF2 protein cannot be seen. The length of the protein markers is shown at the right side of the panels.

Precipitation. Tumour cells were lysed and used for immunoprecipitation with antibody A19. Detection of the NF2 proteins was with the same antibody. The results of this analysis are shown in Figure 2B. As a control Figure 2B shows the position of the wild-type NF2 protein transiently expressed in monkey COS cells. In the lane with the immunoprecipitated MN121 sample a band migrating at the position of full-length NF2 is visible, in addition to the bands of the heavy and light chains of the precipitating antibody and a slower migrating band of unknown identity. However, no truncated mutant protein of the expected size can be detected in tumour MN121.

Presence of the G→A mutation in other members of the NF2 family

The G→A transition detected in PBL DNA from case 2 results in the loss of a BfaI site and creates a new MaeII site. Both these restriction enzymes were used to detect the mutation in other members of the family and the results are shown in Figure 3. Two fragments resulting from MaeII digestion were found in the DNA extracted from peripheral blood lymphocytes of cases 2 and 3, together with the undigested band from the wild-type allele. Similarly, two MaeII fragments were also found in DNA extracted from meningiomas from cases 1 (MN60) and 2 (MN121). In the DNA of case 4 no new MaeII sites or loss of the BfaI site could be detected (Fig. 3). This individual was born in 1938 and has so far not complained of any symptoms reminiscent of NF2, although he has not been tested in a clinical setting. In addition, the G→A transition could not be found in control DNAs extracted from peripheral blood lymphocytes of a series of 50 independent individuals. These results suggest that the G→A transition is indeed the causative mutation in this family. Case 5 was seen for genetic counselling because she wanted to be informed about her carrier status. She was tested according to a presymptomatic counselling protocol. Her DNA was screened using RFLP analysis. She had a wild-type genotype (results not shown).

DISCUSSION

In this paper we describe a mutation which creates a splice branch point sequence in an intron of the NF2 gene. Due to the presence of consensus splice acceptor and donor sites downstream of this mutation, intron sequences are spliced into the NF2 mRNA. Mutations affecting a branch point sequence are not frequently encountered and only two cases have been described so far. Putnam et al. (3) describe a case of congenital contractural arachnodactyly (CCA) in which an A→G mutation destroyed the
invariant A residue of the branch point preceding exon 29 of the FBN2 gene, resulting in reduced usage of the exon. In a family with X-linked hydrocephalus (HSAS) an A→C change destroys
the branch point of exon R (2). This results in omission of the exon from the mRNA or usage of an alternative splice acceptor site 69 nt upstream of the exon. In addition to this, normal splicing of the mutant allele was also observed. In the affected members of the NF2 family described here the reverse is found: a genuine branch point sequence (-TTCTAAC-) is created and 106 bp of intron sequence are spliced into the mRNA between exons 5 and 6. To our knowledge this is the first time such a mutation has been found in a human hereditary disorder.

Mutations that lead to a putative truncated protein are predominantly found in families with the severe form of NF2. In contrast, families with the mild form more often have a missense mutation (9 and references therein; 12,13). A special class is formed by the patients in whom a splice site mutation is detected; these may be either of the mild or severe phenotype. A possible explanation of the latter finding is that the mutation may not always exclusively lead to a mutant transcript but that some degree of normal splicing may still be possible. However, the mRNA and proteins of the splice site mutations have not been investigated. MN121 contains both mutant and considerable amounts of wild-type transcript, as judged from the relative intensities of bands obtained in RT-PCR experiments (results not shown). In the tumour LOH of the wild-type allele was observed (16) and densitometry of the RFLP data suggests that the tumour sample contained 80–90% tumour cells (results not shown; non-tumour cells are presumably derived from blood vessels).

Thus we presume that a considerable fraction of the wild-type transcript originates from the tumour cells and was produced by normal splicing of the mutant transcript. Detection of full-length NF2 protein in the tumour lysate also supports this hypothesis. Therefore, in this family the occurrence of some degree of normal splicing may explain the relatively mild phenotype.

The nucleotide sequence of the mutant cDNA predicts that translation of this alternative mRNA stops within the insert at a stop codon located at 31 amino acids within the 106 nt fragment. These predictions were confirmed by western blotting of COS cells transfected with a NF2 expression vector containing the MN121 mutation and in vitro transcription/translation of this construct. By both analyses we demonstrate formation of a smaller truncated NF2 protein. In whole cell lysates of tumour MN121 the 23 kDa mutant NF2 protein could not be detected, in contrast to full-length wild-type protein. This suggests that the truncated 23 kDa protein is unstable. It has been suggested by several investigators that a mutation leading to a truncated NF2 protein may display a dominant negative effect on the product of the wild-type allele (9,12,13). However, so far there is no proof for this hypothesis. The data presented in this paper show that we were unable to detect the truncated protein, even when considerable amounts of mutant transcript were present. Therefore it seems that, at least in this case, a dominant negative effect of the mutation is unlikely.

Approximately half of all NF2 patients are severely affected (5). However, 80% of the identified mutations are in patients with the severe phenotype. This implies that there is a bias towards detection of mutations that lead to the severe form of the disease (9,12,13). In the group of patients described by Parry germline mutations were detected in only 36% of families with a mild phenotype. Therefore, it is likely that many of the mutations in mildly affected families are within introns and affect splicing of the transcript to some extent. In the family discussed in this paper a mutation affecting splicing was revealed that cannot easily be discovered using SSCA on genomic DNA with exon flanking primers. This will also hold for other similar mutations, because it is practically impossible to screen each intron of the entire gene.

The optimal approach to detect mutations like these is by SSCA on cDNA. A problem with this approach is that RNA isolated from lymphoblasts cannot be used for such assays, probably because of low or absent expression of the gene in these cells (13). The NF2 gene is, however, strongly expressed in skin (17). Thus an alternative and perhaps better approach for mutation detection in NF2 families would be to use RNA isolated from skin biopsies.

**MATERIALS AND METHODS**

**Patients**

The patients mentioned here are members of a family that was described by Delleman et al. (18) as having the central form of Von Recklinghausen disease. Figure 2A depicts the updated pedigree. This family is now diagnosed as having NF2, using the current NIH criteria for diagnosis of NF1 and NF2 (4). The first generation consisted of three affected and four unaffected sibs. The two affected brothers from this generation developed multiple meningiomas and died at ages 70 and 64. The affected sister died at age 43. Upon autopsy, multiple meningiomas and bilateral acoustic neuromas were discovered. The second generation members are descendants of one of the brothers and comprise three affected (and as far as is known) three unaffected sibs.

**Case 1.** A female patient (FIII, 4 in 18), born in 1942. From 1967 multiple intracranial and intraspinal meningiomas were discovered. Several of these were removed by surgery during the following years. The patient died in 1991.

**Case 2.** Sister of case 1 (FIII, 2), born in 1936. In 1972 a bilateral focal cord paresis and a right-sided radiculopathy of L4 and L5 were diagnosed, for which, at that time, no explanation could be found. In 1979 she presented with hearing loss. A CT scan showed a right cerebellar pontine angle tumour besides multiple supratentorial high density lesions suggestive of meningiomas. The cerebellar pontine angle tumour was partially removed: histology, fibrous meningioma. When the tumour recurred in 1992 it was totally removed. The other tumours have not shown progressive growth and the clinical condition of the patient has remained stable.

**Case 3.** Brother of case 1 (FIII, 5), born in 1944. At age 22 a meningioma was removed from the optic nerve sheath. At present he has a harsh voice due to unilateral cord paralysis. Because of an intrathoracal tumour detected in 1993, he is slightly dyspneic. Since 1994 his visual acuity has been deteriorating due to cataracts. In 1997 he was still able to work.

**Case 4.** Brother of case 1, born in 1938. Without symptoms of NF2.

**Case 5.** Daughter of case 3. Was healthy at age 19, although she complained of dizziness.

**NF2 mutation detection**

For the detection of NF2 mutations in genomic DNA, DNA was extracted using routine procedures (19) from tumours MN60...
Fornerod precipitations were performed essentially as described by constructs were transfected into COS cells by electroporation conditions described by the manufacturer. Samples were methionine (10 mCi/ml; Amersham, Little Chalfont, UK) under glycerol and electrophoresed at 8 W at 4 °C × 121B (CACTGCAAACAGCTCAA TTCC, nt 399–379 in Fig. 1) and 5aII (AA TCTCCCAGTAAACAGTGT, nt 192–211 in Fig. 1) and → sequencing kit (Pharmacia, Sweden). For detection of the G → A transition in the family members 2–5 and control individuals. Primers flanking the exons of the NF2 gene were kindly supplied by Dr J.F.Gusella and Jacoby are acknowledged for their gift of the NF2 exon flanking primers (15) was used to amplify the region containing the intron sequence. The PCR fragment was subcloned into pcM™II (TA Cloning Kit; Invitrogen) and the 5′-part of this fragment was sequenced using a T7 DNA sequencing kit (Pharmacia, Sweden). For detection of the G → A transition in the family members and control DNAs the primers 5aII (AAATCTCCAGTAAACAGTGT, nt 192–211 in Fig. 1) and 121B (CACTGCAACAGCTCAAATTC, nt 399–397 in Fig. 1) were used to amplify a 206 bp DNA fragment. After digestion of the radioactive PCR fragment with the restriction enzyme BfaII or MaeII samples were analysed on 12.5% polyacrylamide gels and exposed to Kodak XAR-5 films.

Construction of a mutant cDNA expression construct

The forward primer A1/5′ (CATGGCCGGGCATCGTCCTCC, nt 219–240, relative to the start codon) in combination with the reverse primer A3/3′ (CATAAATAGATCATGGTCCCGAT, nt 1142–1119) (14), flanking the insertion in the NF2 transcript of MN121, were used to amplify the cDNA of MN121. The 1.0 kb PCR product of the primer combination was cloned into pcM™II, using the TA Cloning Kit (Invitrogen). A 0.475 kb BgII/Bgl II fragment was isolated and subcloned into a full-length NF2 cDNA construct, replacing the original BgII fragment. Clones with a properly oriented BgII fragment were selected by restriction analysis and recloned into a pcDNA3 mammalian expression vector. The insert of the thus obtained pR121 clone was sequenced using a sequence kit (Pharmacia, Sweden).

Detection of mutant and wild-type NF2 protein

In vitro transcription/translation was performed using the TnT Coupled Reticulocyte Lysate System (Promega, Madison, WI) with T7 primer, 1 μg plasmid DNA and 2 μl [35S]-labeled methionine (10 mCi/ml; Amersham, Little Chalfont, UK) under conditions described by the manufacturer. Samples were analysed using SDS–PAGE and autoradiography. The expression constructs were transfected into COS cells by electroporation (250 V, 500μF). After 48 h the transfected cells were washed with PBS, harvested by scraping with a rubber policeman, lysed in reducing sample buffer and used for western blotting. Immuno-precipitations were performed essentially as described by Fornerod et al. (20). Briefly, MN121 tumour tissue was homogenized by mincing in immunoprecipitation buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 % Nonidet P40, 5 mM EGTA, 5 mM EDTA, 15 mM MgCl2, 1 mM dithiothreitol, 0.1 mM NaVO4, 0.1 mM NaF) supplemented with protease inhibitors. The crude lysate was centrifuged and filtered through a 45 μm filter. After pre-clearing, specific antibody (A19, sc331; Santa Cruz Biotechnology, Santa Cruz, CA) and 40 μl of a 50% slurry of Sepharose–protein A were added. After overnight incubation at 4 °C the beads were spun down and washed four times in immunoprecipitation buffer. The beads were resuspended in reducing sample buffer and the sample subjected to electrophoresis and blotting as described previously (17). After blocking, the NF2 protein was detected by incubating the blots with antibody A19, followed by a secondary biotinylated goat anti-rabbit antibody and peroxidase-conjugated streptavidin. Bands were visualized by enhanced chemiluminescence and exposure to film.

ACKNOWLEDGEMENTS

We are grateful to the members of the family described in this report for their collaboration. We also wish to thank Dr N.T.Tijmes for help with contacting some members of the family and Dr M.Mannens for pre-symptomatic testing of case 5. Drs Gusella and Jacoby are acknowledged for their gift of the NF2 exon flanking primers. This work was supported by the Dutch Cancer Society.

REFERENCES


