Fine Mapping of the Locus for Nevoid Basal Cell Carcinoma Syndrome on Chromosome 9q

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The nevoid basal cell carcinoma syndrome (NBCCS) is an autosomal dominant disorder, characterized by predisposition to multiple early basal cell carcinomas of the skin and several other tumors as well as frequent occurrence of developmental anomalies. The gene has previously been mapped to chromosome 9q22 and is believed to function as a tumor suppressor. We have applied linkage and haplotype analysis to four Swedish nevoid basal cell carcinoma syndrome families to refine the localization of the nevoid basal cell carcinoma syndrome gene. Information from critical recombinants localizes the gene proximal of marker D9S287, which in combination with analysis of loss of heterozygosity in a hereditary cardiac fibroma has allowed us to define a minimal candidate region of 1 Mb or less for the nevoid basal cell carcinoma gene flanked by the markers D9S280 and D9S287 in the 9q22.3 area. Key words: Gorlin syndrome; gene mapping; linkage analysis.

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The nevoid basal cell carcinoma syndrome (NBCCS) is an autosomal dominant disorder initially reported over 100 years ago (1) and fully described by Gorlin & Goltz (2, 3). NBCCS is characterized by complete penetrance but markedly variable expression (4) and entails predisposition to multiple basal cell carcinomas (BCCs) of the skin and various other tumors such as ovarian and cardiac fibromas and desmoplastic medulloblastomas (5-7). Abnormal embryonic development is commonly seen among affected individuals, implying that the NBCCS gene plays an important role in normal development as well as in the control of cell growth and differentiation.

The major features seen in NBCCS patients are multiple BCCs, palmar and plantar pits, odontogenic keratocysts and calcification of falx cerebi occurring in more than 75% of reported cases (6, 8). Minor clinical features mainly include developmental and skeletal malformations such as spina bifida occulta, frontal bossing, macrocephaly, strabismus, cleft palate, bifid ribs, ectopic calcification and general overgrowth.

No major cytogenetic constitutional abnormalities associated with the syndrome have been described (9, 10). The estimated minimum incidence is 1 in 55,000 in the United Kingdom (6) and as high as 1 in 5 among patients that develop BCC before the age of 19. Through linkage analysis the NBCCS gene has been mapped to chromosome 9q22.3-31 in North American, European and Australian families (5, 11-13). Further mapping has limited the candidate genomic region to a 2 Mb interval between the loci D9S196 and D9S180 (14, 15) (Fig. 1). Linkage to the same area has been reported in two African-American pedigrees (16), despite clinical manifestations of the disease being quite different in these families (14). Interestingly, the genes for three other cancer predisposition syndromes — multiple self-healing squamous epitheliomata (ESS1), Fanconi complementation group C (FACC) and xeroderma pigmentosum complementation group A (XPAC) — have been assigned to the same chromosomal region (17-19). The latter two genes have been excluded as candidate genes because of differences in phenotype and mode of inheritance of the associated disorders and by the lack of mutations in NBCCS patients (20).

Ionizing radiation has been reported to precipitate the development of BCCs in NBCCS patients (8), and most tumours usually develop on sun-exposed body sites (8, 21, 22), consistent with an important role of these environmental factors in skin tumour induction.

The NBCCS gene is believed to function as a tumour suppressor gene, based on frequent loss of heterozygosity (LOH) in the 9q22.3 region affecting the wild-type allele derived from the unaffected parent in hereditary tumours (5, 23). Furthermore, deletions in the same genomic region are seen in 50–75% of sporadic BCCs (5, 8, 13, 24, 25), indicating

Fig. 1. Map of the NBCCS candidate genomic region. Arrows denote identified critical recombinants at D9S196, D9S287 and D9S180.

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that the NBCCS gene may play an important role in the development of the majority of BCCs.

To refine the localization of the NBCCS gene, we used linkage and haplotype analysis of Swedish NBCCS families. The results from these analyses together with LOH analysis of a hereditary cardiac fibroma suggest a minimal candidate region located between markers D9S280 and D9S287.

MATERIALS AND METHODS

Families and diagnostic criteria

Twenty-four patients with NBCCS from 11 different families were recruited through the Department of Dermatology, Karolinska Hospital. Four families were suitable for linkage and haplotype analysis, having 2 or more affected members. The final sample comprised 48 subjects, 13 affected family members and 35 non-affected. To be classified as affected, individuals had to meet one of the following criteria: at least two major features of NBCCS, or alternatively one major feature or two minor features in combination with having one affected first-degree relative (6). All the patients and relatives were classified based on medical history (including jaw cysts, BCCs, skeletal problems, enlarged head circumference early in childhood) and clinical examination by the same dermatologist (ABU). The entire body was screened for BCCs and the occurrence of palmar and plantar pits; frontal bossing, hypertelorism or typical skeletal anomalies were also noted. Radiographic examination was performed of the skull to assess calcification of the falx cerebri. Panoramic and X-ray of vertebrae column, costal ribs and hands were also performed. Six of the patients (individual II-1 in family 1, individuals III-2 and III-3 in family 2 and 3 of the patients in family 4) were not examined for intracranial calcifications. Among the adult patients, 4 had four major features, 3 had three major features, 2 had two major features and all had multiple minor features. Four affected children presented with minor features. Family 1 showed affected subjects in three generations, the others in two. In family 2 patient III-3 died shortly after birth from a cardiac fibroma. All the patients' medical, clinical and radiographic data are summarized in Table I.

DNA extraction

Genomic DNA was extracted from peripheral leukocytes using a fast method for high-quality genomic DNA (26). In addition, in one deceased family member genomic DNA was extracted from normal and cardiac fibroma archival paraffin-embedded tissue using Nucleon™ (Scotlab Ltd, Strathclyde, UK). The paraffin sections contained more than 70% tumour cells. The extraction was performed according to Ma et al. with small modifications (27). Briefly, prior to DNA extraction, paraffin was removed by adding 100 µl NIB buffer (0.45% NaPO4, 0.45% Tween 20, 50 mM KCl, 10 mM Tris pH 8.3, 1.5 mM MgCl2 and 100 µg/ml gelatine) to the sample and boiling for 20 min followed by 10 min centrifugation at 13,000 rpm. The supernatant was then removed and used for further processing.

Polymerase chain reaction

Nine microsatellite markers and one restriction-fragment-length polymorphism present in the Fanconi anaemia complementation group C gene located to chromosome 9q22.3 were used to genotype DNA from all the patients and the unaffected relatives using PCR amplification (primer sequences are given in Table II). We used a laser-based automated DNA fragment analyser to size PCR products (28, 29). The fluorescent-labelled primer required in this method was purified successively through a PD 10 column (Pharmacia, Milton Keynes, UK), followed by cartridge purification on an OPC column (Applied Biosystems, Foster City, California, USA) and finally precipitated in ethanol, dissolved in distilled water and stored at -20°C. Non-fluorescent primers required no purification before use. Genomic DNA (100 ng) was amplified in 10-µl reactions containing 10 mM Tris pH 9.0, 50 mM KCl, 0.1% TritonX-100, 2.0 mM MgCl2, 200 µM dNTPs, 5.0 pmol of each primer (10 pmol in the FACC reactions) and 0.75 units of Taq DNA polymerase (Promega, Scandinavian Diagnostic Services, Falkenberg, Sweden). Samples were amplified using a DNA Thermal Cycler 480 (Perkin Elmer AB Sundbyberg, Sweden) with denaturation and extension temperatures of 94°C and 72°C for 1 min, with variable annealing temperatures (Table II) and with a final 10-min extension. PCR products were separated on a 6% polyacrylamide denaturing gel in TBE buffer using a Model ABI 373A automated fluorescent DNA sequencer (Applied Biosystems, Foster City, California, USA). The fluorescent gel data collected during the run were automatically analyzed by the GENESCAN 672 software (Applied Biosystems, Foster City, California, USA). Each fluorescent peak was quantified in terms of size, height and area. All analyses of critical individuals were repeated at least three times.

Genetic analysis

NBCCS was modelled as a rare autosomal dominant trait with complete penetrance and a gene frequency of 1x10⁻³ (6). The sporadic rate is estimated to 0.001 in individuals over 33 years. Five liability classes were used, giving different penetrance with varying age (15). Two-point analyses were conducted using the MLINK program.

<table>
<thead>
<tr>
<th>Family</th>
<th>Patient</th>
<th>Age (years)</th>
<th>Year of diagnosis</th>
<th>No of BCCs</th>
<th>Major features</th>
<th>Minor features</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>II-1</td>
<td>57</td>
<td>41</td>
<td>Multiple</td>
<td>No</td>
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<tr>
<td>1</td>
<td>III-2</td>
<td>31</td>
<td>15</td>
<td>Multiple</td>
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<td>Multiple</td>
</tr>
<tr>
<td>1</td>
<td>IV-2</td>
<td>8</td>
<td>4</td>
<td>0</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>1</td>
<td>IV-1</td>
<td>1.5</td>
<td>Newborn</td>
<td>0</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>III-1</td>
<td>47</td>
<td>29</td>
<td>Multiple</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>III-2</td>
<td>18</td>
<td>Newborn</td>
<td>Multiple</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>III-3</td>
<td>15</td>
<td>Newborn</td>
<td>0</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td>III-1</td>
<td>67</td>
<td>30</td>
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<tr>
<td>3</td>
<td>III-2</td>
<td>25</td>
<td>13</td>
<td>0</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>4</td>
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<td>60</td>
<td>50</td>
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<td>No</td>
</tr>
<tr>
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<td>42</td>
<td>30</td>
<td>One</td>
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<td>Yes</td>
</tr>
<tr>
<td>4</td>
<td>III-2</td>
<td>32</td>
<td>32</td>
<td>One</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

ND = not done; a = deceased.
Table II. Details of the 10 PCR based markers used

<table>
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<tr>
<th>Microsatellite marker</th>
<th>Heterozygosity</th>
<th>Number of alleles</th>
<th>Annealing temperature</th>
<th>Primer sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>D9S153</td>
<td>0.76</td>
<td>7</td>
<td>61°C</td>
<td>GCA GAA TGT CCA AAA CTC A</td>
</tr>
<tr>
<td>D9S122.PCR2</td>
<td>0.67</td>
<td>4</td>
<td>60°C</td>
<td>TTA TGG GAC CCC AAA TGG ACT A</td>
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<tr>
<td>D9S151</td>
<td>0.76</td>
<td>10</td>
<td>65°C</td>
<td>GCG AGA CTG TCT ACA CAC AC</td>
</tr>
<tr>
<td>D9S197</td>
<td>0.67</td>
<td>7</td>
<td>59°C</td>
<td>CGA GTC CAC GTG GTT TGT</td>
</tr>
<tr>
<td>D9S196</td>
<td>0.65</td>
<td>5</td>
<td>59°C</td>
<td>GAG GGT GCA GGG GGA CTG CC</td>
</tr>
<tr>
<td>D9S280</td>
<td>0.63</td>
<td>5</td>
<td>66°C</td>
<td>TCC CTT CCT GGG CTG CTA GG</td>
</tr>
<tr>
<td>FACC</td>
<td>0.54</td>
<td>2</td>
<td>55°C</td>
<td>TGG CTT GGA TTA GGA AC</td>
</tr>
<tr>
<td>D9S267</td>
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<td>63°C</td>
<td>ACC ACA CAC GCA CGT TT</td>
</tr>
<tr>
<td>D9S180</td>
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<td>4</td>
<td>61°C</td>
<td>AGT CCT GCT TTA GGA AAC</td>
</tr>
<tr>
<td>D9S272</td>
<td>0.79</td>
<td>9</td>
<td>58°C</td>
<td>ACT GAA TTT GGA GAA CAC</td>
</tr>
</tbody>
</table>

RESULTS

Clinical traits

The majority of patients showed several signs and symptoms frequently reported in NBCCS (6, 8) (Table I). The mean age of onset for keratocysts was 18 years and for BCCs 29 years. However, three children presented with their first BCCs at ages 4, 12 and 14. Pitting, in most cases both palmar and plantar, was found in 89% of the patients. Three children (2 boys and an 8-year-old girl) showed no pits. Calcification of falx cerebri was present in all the adults examined, but absent in all children. A cardiac fibroma was the only non-BCC neoplasm identified. A male patient in family 6 died at the age of 32 years due to multiple untreated BCCs. According to Shanley et al. (8) the new mutation rate in NBCCS ranges between 14% and 81%. In support of a high frequency of de novo mutations, our results suggest a new mutation rate of 50%.

Linkage and haplotype analysis

The NBCCS gene region has previously been located to 9q22.3 between the microsatellite markers D9S180 and D9S196 (14, 15). We carried out pairwise linkage analysis between NBCCS and the microsatellite markers D9S151, D9S196, D9S280, D9S287 and D9S180 located to chromosome 9q22.3. Data compatible with linkage were found for D9S151 (Zmax = 2.54, \( \theta = 0.00 \)), D9S196 (Zmax = 1.58, \( \theta = 0.00 \)) and D9S280 (Zmax = 1.78, \( \theta = 0.00 \)). All four families showed positive lod scores for all markers. However, overall negative lod scores were obtained for D9S287 and D9S180 due to the presence of affected recombinants in families 1 and 2. In addition, the results of haplotype analyses in the four families were consistent with a location of the NBCCS gene to the 9q22.3 area.

Critical recombinants and LOH analysis

Among the 48 individuals in our study we identified two recombinants, using haplotype analysis. One recombination between NBCCS and D9S180 occurred in an affected member, III-3, of family 2 (Fig. 2). This girl, having an affected father, died shortly after birth from a cardiac fibroma. In addition she presented with macrocephaly and frontal bossing. These results place the NBCCS gene centrometrically of D9S180. The second recombination was between NBCCS and D9S287 in a three-generation family (Family 1, VI-1, Fig. 2). This 1.5-year-old girl, also with an affected father, presented as newborn with enlarged calvaria (far above the 97.5 percentile), strabismus and frontal bossing. All features have remained up to the present time. A CT scan taken at 1 year of age showed enlarged lateral ventricles. Based on these findings she is classified as affected, which places the NBCCS gene centrometrically of D9S287.

The occurrence of a cardiac fibroma in an affected patient (III-3) in family 2 offered the possibility of LOH analysis to further data relevant to the sublocalization of the NBCCS gene. LOH was observed for D9S287 by the naked eye with an allele ratio of 0.10, whereas no sign of LOH was seen for D9S280 (allele ratio 0.89) or D9S196 (allele ratio 0.97) (Fig. 3). Marker D9S180 was not informative. LOH was also seen for the centromeric marker D9S122, having an allele ratio of 0.18. The allele lost in both cases was derived from the non-affected parent.

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**DISCUSSION**

NBCCS is associated with a broad spectrum of clinical traits, which are to a large extent linked to skeletal anomalies and the deregulated growth of epithelial cells (6, 8). In general, the clinical findings in our Swedish family material were typical of the syndrome and very similar to previous observations, with frequent occurrence of palmar and plantar pits, macrocephaly and BCCs. The mean age of onset of BCCs (29 years) is in the same range as reported from England (6) but higher than the corresponding age (20.3 years) observed in an Australian population (8). This difference may reflect less exposure to ultraviolet radiation in European populations. Interestingly, we found three children who presented with BCC at the ages of 4, 12 and 14 years. These children have no known history of radiation therapy or exposure to other carcinogenic agents, suggesting that BCCs may, at least in some cases, be initiated very early, possibly as early as the fetal stage. Such early onset is implicated in other tumour forms associated with NBCCS, such as medulloblastoma and cardiac fibroma, (6), and was also found in one of our cases where infantile death was caused by the presence of a cardiac fibroma.

The results of linkage and haplotype analysis were compatible with localization of the NBCCS gene to 9q22.3 in the four families. Two critical recombinants (Fig. 2) were identified, providing information relevant to the sublocalization of the NBCCS gene. A recombination between the NBCCS gene and the D9S180 marker occurred in a girl who died shortly after birth (III-3). This girl’s classification as affected was based on her having clearly affected first-degree relatives, enlarged calvaria and cardiac fibroma, a tumour type associated with NBCCS (3, 6). A similarly affected recombinant has previously been reported by Wicking et al. (15) and consequently places the NBCCS gene proximal to D9S180 assuming the marker order, cen–D9S151/D9S12–D9S196–D9S280–D9S287–D9S180–tel, suggested at the recent Chromosome 9 meeting (32). The second recombination occurred between NBCCS and the marker D9S287 in a girl, 1.5 years of age at the time of diagnosis. She showed no major features of NBCCS.
(6) and we thus classified her as affected. The new data indicating a location of the NBCCS gene proximal to the marker D9S287 are contrary to a recent report based on deletion mapping in BCCs, suggesting that the NBCCS gene may lie distal to D9S287 (33), but are supported by Farndon et al. (34), who describe a recombination between NBCCS and D9S287 in an unaffected family member. Since the NBCCS gene is likely to be a tumour suppressor gene, analysis of LOH in tumour tissue may yield further information relevant to the localization of the gene. LOH affecting the 9q22 genomic area has previously been described for familial BCCs, ovarian fibromas, medulloblastomas and recently for odontogenic keratocysts (5, 7, 35). Our analysis of a cardiac fibroma from an affected patient demonstrated for the first time the loss of genetic material from the wild type allele on 9q22.3 in this tumour type, further strengthening the evidence for the NBCCS gene being a tumour suppressor. Of even greater interest was the finding of a deletion breakpoint within the candidate area such that marker D9S287 was lost but not D9S260 or D9S196. These results, combined with data from the literature showing recombination between marker D9S196 and the NBCCS gene, give a minimum candidate region between markers D9S260 and D9S287. The exact size of this region is at present not known but is estimated to be 1 Mb or less (32).

In summary, combined information from critical recombinants and analysis of LOH in a hereditary tumour suggest a minimal candidate region of 1 Mb or less for the NBCCS gene located between markers D9S260 and D9S287 on chromosome 9q22.3. This is a significant narrowing of the possible region of the NBCCS gene. We are currently testing new markers between D9S260 and D9S287 in the recombinants and the cardiac fibroma to further refine the location of the NBCCS gene on chromosome 9q22.3.

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REFERENCES


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The new data on the nevoid basal cell carcinoma syndrome (NBCCS) is based on the results of a study by Farron et al. (1992) and the findings by Rein SA, et al. (1993). The NBCCS gene is located on chromosome 9q22-32.3, within the same region as the von Hippel-Lindau tumor syndrome. The loss of heterozygosity (LOH) analysis revealed a loss of heterozygosity at this chromosomal region. The LOH analysis is a critical tool for the identification of candidate genes involved in the disease. The data suggests that the NBCCS gene is not associated with any known genes for inherited disorders. Further studies are needed to confirm the role of this region in the development of nevoid basal cell carcinoma syndrome.


34. Farron PA, Morris DJ, Hardy C, McConville CM, Weissbach J, Kilpatrick MW, et al. Analysis of 133 meioses places the genes for nevoid basal cell carcinoma (Gorlin) syndrome and Fanconi anemia group A in a 2.6 cM interval and contributes to the fine map of 9q22.3. Genomics 1994; 23: 486–489.