Chromosome 2 (2p16) abnormalities in Carney complex tumours

L Matyakhina*, S Pack*, L S Kirschner, E Pak, P Mannan, J Jaikumar, S E Taymans, F Sandrini, J A Carney, C A Stratakis

Carney complex (CNC, OMIM 160980) is an autosomal dominant syndrome characterised by spotty skin pigmentation, cardiac, skin, and breast myxomas, and a variety of endocrine and other tumours. The disease is genetically heterogeneous; two loci have been mapped to chromosomes 17q22-24 (the CNC1 locus) and 2p16 (CNC2). Mutations in the PRKAR1A tumour suppressor gene were recently found in CNC1 mapping kindreds, while the CNC2 and perhaps other genes remain unidentified. Analysis of tumour chromosome rearrangements is a useful tool for uncovering genes with a role in tumorigenesis and/or tumour progression. CGH analysis showed a low level 2p amplification recurrently in four of eight CNC tumours; one tumour showed specific amplification of the 2p16-p23 region only. To define more precisely the 2p amplicon in these and other tumours, we completed the genomic mapping of the CNC2 region, and analysed 46 tumour samples from CNC patients with and without PRKAR1A mutations by fluorescence in situ hybridisation (FISH) using bacterial artificial chromosomes (BACs). Consistent cytogenetic changes of the region were detected in 40 (87%) of the samples analysed. Twenty-four samples (60%) showed amplification of the region represented as homogeneously stained regions (HSRs). The size of the amplicon varied from case to case, and frequently from cell to cell in the same tumour. Three tumours (8%) showed both amplification and deletion of the region in their cells. Thirteen tumours (32%) showed deletions only. These molecular cytogenetic changes included the region that is covered by BACs 400-P-14 and 514-O-11 and, in the genetic map, corresponds to an area flanked by polymorphic markers D2S2251 and D2S2292; other BACs on the centromeric and telomeric end of this region were included in varying degrees. We conclude that cytogenetic changes of the 2p16 chromosomal region that harbours the CNC2 locus are frequently observed in tumours from CNC patients, including those with germline, inactivating PRKAR1A mutations. These changes are mostly amplifications of the 2p16 region, that overlap with a previously identified amplicon in sporadic thyroid cancer, and an area often deleted in sporadic adrenal tumours. Both thyroid and adrenal tumours constitute part of CNC indicating that the responsible gene(s) in this area may indeed be involved in both inherited and sporadic endocrine tumour pathogenesis and/or progression.
Gene amplification may be detected at the cytogenetic level either extrachromosomally, as double minute chromosomes (DMs) (small, dot-like chromosome masses that can contain thousands of copies of an oncogene) or intrachromosomally as homogeneously staining regions (HSRs) that are frequently in tumour DNA. By CGH, a technique that allows the detection of large changes (DMs) (small, dot-like chromosome masses that can contain either extrachromosomally, as double minute chromosomes or intrachromosomally as HSRs), suggesting that an oncogene may lie at this chromosomal location.

**MATERIALS AND METHODS**

**Subjects and tissues**

We studied 46 tumours from familial (34) and sporadic (12) cases of CNC. The patients and the histology of their tumours are listed in table 2. All subjects gave informed consent for the study. The first column of the table provides the designated CAR code for each family assigned by the investigators. The second column shows the histological diagnosis of the tumour studied. The third column lists the genetic locus (CNC1 versus CNC2). “Sporadic” indicates a case with no known family history and presumably de novo mutation of the gene responsible for CNC in this subject. The fourth column lists the results of PRKAR1A mutation analysis. The fifth column presents the CGH results. PPNAD=primary pigmented nodular adrenocortical disease.

**Table 1: CGH abnormalities in CNC tumours**

<table>
<thead>
<tr>
<th>Case</th>
<th>Kindred</th>
<th>Tumour pathological diagnosis</th>
<th>Locus</th>
<th>Mutation of PRKAR1A</th>
<th>CGH results</th>
<th>Gains</th>
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<tbody>
<tr>
<td>1</td>
<td>CAR01</td>
<td>Ovarian cyst</td>
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<td>c.578delGT</td>
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<td>CAR110</td>
<td>Facial myxoma</td>
<td>CNC1</td>
<td>c.765C&gt;T</td>
<td>2p16-23; 5q (middle)</td>
<td>6q22-pter; 10p; 9qter</td>
<td>11</td>
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<tr>
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<td>CAR110</td>
<td>Thyroid carcinoma</td>
<td>CNC1</td>
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<td>PPNAD</td>
<td>Exon 2058/2A-G</td>
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**Fig 1** displays these and their location on the 2p16 genomic map, based on newly identified BACs used in the experiments. The current map consists of over 100 large insert clones and contains over 150 polymorphic and non-polymorphic STS. It covers approximately 10 Mb of physical distance between the markers D2S378 at the centromeric and D2S391 at the telomeric end. The process of the work, 41 new STSs were generated and placed on the map (Kirscher and Stratakis, unpublished data).

Brieﬂy, the isolation of new BACs and STS/EST content mapping was done as follows: polymerase chain reaction (PCR) analyses of all DNA samples were carried out under standardised conditions consisting of 10 µl reactions that contained 1 × PCR buffer 1 (Perkin-Elmer, Foster City, CA), 200 µmol/l each dNTP, 0.6 µmol/l of forward and reverse primers, 50 ng DNA, and 0.5 U Taq polymerase. The reaction mixtures also contained 12% sucrose and 180 µmol/l cresol red to facilitate direct loading of the samples after PCR. Cycling reactions were performed with an initial five minute denaturation step at 95°C, followed by 30 cycles of 95°C for one minute, 56°C for one minute, and 72°C for one minute. Primer sets that did not

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generate reproducible bands from whole human genomic DNA were not considered for further use. For EST/STS content mapping, BAC DNAs were prepared from 5 ml overnight cultures, using commercially available kits (Puregene, Gentra Systems, Minneapolis, MN) and spin miniprep columns (Qiagen Corp, Valencia, CA), respectively.

To obtain BAC end sequences, DNA samples from individual clones were purified using a modified alkaline lysis procedure.19 DNA purified in this way was sequenced directly using the T7 primer or an SP6 end primer (AAGGGTTTGGG) with Big-Dye Terminators (Perkin-Elmer, Foster City, CA). BAC end sequences were then submitted to the Whitehead Institute’s Primer3 software (http://www.genome.wi.mit.edu/genome_software/other/primer3.html) to select 20 base pair (bp) PCR primers that were not complementary to known repetitive sequences.

Pulsed field gel electrophoresis was used to analyse the size of BAC inserts. BAC DNA was prepared for sequencing as described above and digested in solution with NotI before electrophoresis. All pulsed field analyses were performed on a CHEF Mapper II apparatus (Biorad Laboratories, Hercules, CA), using programs recommended by the manufacturer to resolve DNA fragments of the appropriate size.

Fluorescence in situ hybridisation (FISH)

FISH was performed as described elsewhere.31 In brief, DNA was labelled by digoxigenin-11-dUTP or biotin-11-dUTP by nick translation followed by anti-dig-rhodamine or avidin-FITC detection. After precipitation in ethanol in the presence of 50% deionised formamide/10% dextran sulphate/2×SSC (50% Cot-1 fraction of human Cot-1 DNA), the DNA pellet was resuspended in Hybrizol solution (50% deionised formamide/10% dextran sulphate/2×SSC) with a final concentration of 25 mg/ml. For dual colour FISH, the concentration of each probe was 50 mg/ml. After the detection reaction, cells were counterstained with DAPI. Hybridisation signals were analysed using a Leica epifluorescence microscope; fluorescence images were automatically captured on a Photometrics cooled CCD camera (Photometrics Ltd, Tuscon, AZ), using IP Lab Image software (Scanalytics Inc, Fairfax, VA). At least 100 non-overlapping cells with...
2p16 in CNC tumours

Figure 1: Ideogram of chromosome 2 (left) with microsatellite markers (middle vertical line) and BAC clones (right). Polymorphic markers are marked by asterisks; previously undescribed BAC clones are indicated in red. All STSs and remaining BACs have been described by Kirschner et al.

DNA was extracted as described elsewhere. A chromosome 2 specific centromeric α-satellite probe (Vysis) or a painting probe specific for chromosome 2 was also used for chromosome identification.

Statistical analysis
For all analyses, χ² test with Fisher’s correction, when appropriate, was performed using the Statistica software (StatSoft, Tulsa, OK).

RESULTS
CGH
We performed CGH on eight samples of tumours from CNC patients, as a first screening technique to detect possible large scale chromosomal aberrations associated with these lesions. These results are presented in table 1.

The most frequent changes were gains, whereas losses were detected in only five of eight samples. The greatest number of changes were detected in cases 1, 2, and 6 (ovarian cyst, pituitary adenoma, and PPND). Three samples (cases 5, 7, and 8) showed amplification only of a single region. Changes detected in more than one tumour involved losses of 6q and 11, whereas chromosomes 2, 9, 16, 19, 20, and 22 were involved in repeatedly identified gains. In total, 13 chromosomal regions showed amplification, but in six the amplification was seen in only one sample. The most frequent and greatest contiguous change was amplification of 2p, which was detected in four of eight samples. One of the samples had a relatively narrow region of amplification that was defined as containing the 2p16-p23 region.

Identification of new BACs
A total of 12 new BACs, identified by STS to map to the previously described 2p15-21 contig, are presented in fig 2. They complement the existing map, but leave unclosed the known central gap. Their location was confirmed by fiber-FISH (data not shown) and was correlated with known BACs.

Amplification of the 2p16-21 region
In order to define more precisely the region amplified on 2p, we used FISH with BACs from the 2p15-p21 region. Table 2 presents results of FISH on 46 tumour samples. Abnormalities were detected in the 34 tumours studied but only in six of the 12 cell lines; the difference was statistically significant (p<0.05). FISH detected amplification of the region in 24 (60%) of 40 tumours that showed abnormalities (fig 3C-F). Three tumours (8%) showed both amplification and deletion of the region in their cells. To define the extent of the region of amplification, we used dual colour FISH with BAC probes from the centromeric and telomeric borders of 2p16-21. BACs 388-14 and 18-A-5 were not amplified, establishing the centromeric and telomeric boundaries of the amplified region in the tumours above D2S1337 (2p16.1) and below SHGC14952 (2p21), respectively (fig 3B). The borders of this amplicon were flanked by probes b1-p-2 (D2S2378) and b286-c-2 (D2S378), although the sizes of amplicons varied between tumours and between cells in one tumour. For example, an amplicon in cases 15 and 7 was flanked by the BAC probes mentioned above, from the centromeric and telomeric borders of the 2p15-21 region, respectively. This region of amplification included therefore more than 10 Mb of genomic distance, far above and below the CNC2 critical region. In case 32, on the other hand, the amplicon included only BAC 400-P-14 and its most proximal BAC514-O-11; characteristically, however, BAC 514-O-11 was amplified in a much smaller fraction of the cells than BAC 400-P-14.

The level of amplification also varied from sample to sample and frequently within the same tumour (fig 3F). Also, some cells displayed an amplification of one or both alleles. A high level of amplification was detected in only three tumours.
(cases 2, 17, and 33); the majority of the specimens showed low or mid-level amplification counting 4-20 copies of the probe.

Probes displayed a tandem pattern of amplification in the interphase nuclei indicating the presence of HSRs, which were seen as a side by side duplication of dual colour BAC signals (fig 3C, D). Metaphase FISH analysis of a cell line established from a cheek myxoma (case 34) showed cells with differential signal intensity of the 2p16 region (fig 3E).

The region of most consistent amplification was defined by BAC clones 400-P-14, 435-I-1, and 514-O-11 (D2S2251-D2S2292). These BACs were included in the amplicon of all tumours that displayed amplification. They also had a greater copy number than the BACs used in the present study; in one tumour, BACs 400-P-14 and 435-I-1 showed higher level of amplification and in a greater number of cells than BAC 514-O-11, although in all other tumours these three BACs had a similar degree of amplification.

Control experiments for the amplification
FISH analysis with α-satellite probe specific for chromosome 2 centromere detected two copies of the chromosome in all tumours analysed indicating that the cells were diploid. In addition, the 2p16 BACs that showed copy number gain on CNC tumours were hybridised to normal tissues from the same patients; the expected two copies were found in all cells. BAC clones from chromosomes 1, 10, 16, and 22 when hybridised to CNC tumours also showed the expected two signals. Additionally, control experiments included hybridisation of BACs 400-P-14 and 514-O-11 to prostate adenoma tumour cells (fig 4D), breast cancer cell lines HTB22 and HTB30, and pancreatic cancer cell line CRL1240. Two signals of equal intensity were shown in more than 90% of prostate adenoma cells examined. Breast cancer cell lines HTB22 and HTB30 showed equal copy numbers of the BACs and α-satellite probe specific for chromosome 2 centromeres. Pancreatic cancer cells also showed no amplification of BACs 400-P-14 and 514-O-11, although we detected a translocation of the region (data not shown). Taken together, the data allowed us to conclude that the cytogenetic changes of the 2p16 region were specific to CNC.

Deletion of 2p16 region
In addition to amplification of the 2p16-21 region in CNC tumours, we also detected an allelic deletion of the region in 13 tumours (32%); amplification and deletion were seen in three tumours (table 2). Some tumours, primarily those of adrenocortical origin, lost the whole chromosome 2 as shown by FISH experiments with a chromosome 2 specific centromeric α-satellite probe or a chromosome 2 specific painting
[72x222]probe. However, most of the samples showed two copies of chromosome 2 in their cells.

The size of the deleted region in these tumours varied, but it was generally considerably smaller than the region of amplification. It was flanked by BACs 325-M-13 (2p16.3) and 400-P-14 (2p16.1) from the telomeric and centromeric sides, respectively (fig 1). Dual colour FISH with BACs from within the region narrowed the boundaries of the deleted region. For example, FISH of BACs 43-E-9 (red) and 79-H2 9 (green) showed multiple signals of each colour. FISH of BACs 1121-8 and 522-0-16 hybridised to a pituitary adenoma cell (case 17) detected amplification of both BACs. FISH 400-P-14 hybridised to metaphase cells of a short term culture of a cheek myxoma (case 34). A consistent differential signal intensity of chromosome 2 supports amplification of the region 2p16. FISH of BACs 400-P-14 and 514-O-11 (fig 4C) to skin myxoma cells (case 12) showed a deletion of both BACs establishing the minimal size of the deleted region in CNC tumours (fig 2). BAC 435-I-1 that was the nearest one to BAC 400-P-14 (and slightly overlaps with it) on the physical map of the 2p16 region was lost in CNC tumours together with BAC 400-P-14. BAC 112-l-8 that maps more centromeric to BAC-400-P-14 showed two signals in all tumours analysed.

Taken together, these data suggested that the region covered by BACs 400-P-14, 435-I-1, and BAC 514-O-11 most probably contained the CNC2 locus. Based on the sizes of BACs 400-P-14, 435-I-1 and 514-O-11 (160, 125, and 160 kb, respectively) and taking into account the overlapping of BACs 400-P-14 and 435-I-1 on the published map, we can assume that the size of this region is approximately 400-450 kb.

Control experiments for the deletions
Prostate adenoma tumours used as controls showed less than 10% of cells with one signal of BACs 400-P-14 or 514-O-11. FISH with other 2p16 BACs also showed the expected two signals. Breast cancer cell lines HTB22 and HTB30 showed equal copy numbers of these BACs and α-satellite probes that are

Figure 3 Amplification of the 2p16-21 region in CNC tumours. (A) Mapping of the two clones used in this study to chromosome 2 (2p16-21) by FISH on a metaphase spread; the green and red signals correspond to BAC 209-K-4 (D2S1248) and BAC 1-P-2 (D2S2378). (B) An ovarian tumour cell (case 1) hybridised with BACs 18-A-5 (red) and 338-A-14 (green) containing SHGC14952 (2p21) and D2S1337 (2p16.1). These BACs were not amplified in CNC tumours establishing the boundaries of amplified region. (C) A cell from the same tumour as in (B) after hybridisation with BACs 43-E-9 (red) and 79-H2 9 (green) showed multiple signals of each colour. (D) FISH of BACs 1121-8 and 522-0-16 hybridised to a pituitary adenoma cell (case 17) detected amplification of both BACs. (E) FISH 400-P-14 hybridised to metaphase cells of a short term culture of a cheek myxoma (case 34). A consistent differential signal intensity of chromosome 2 supports amplification of the region 2p16. (F) Gluteal myxoma cells (case 44) hybridised with BAC 400-P-14 (red) and α-satellite probe specific for chromosome 2 (green). Cells showed different levels of amplification of BAC400-P-14 (red); in these cells, the centromeric α-satellite probe showed two signals. Some cells were normal, showing two copies of both probes; other cells showed three or four copies of both probes, representing triploid and tetraploid (or dividing) cells.
specific for the centromere of chromosome 2. When possible, normal tissue associated with the CNC tumours was also hybridised with the corresponding BAC; no abnormalities were detected in these cells. Finally, control experiments included hybridisation of BAC clones from chromosomes 1, 4, 16, and 22 to CNC tumours. The expected two signals were detected in more than 90% of cells, suggesting that the observed 2p16 changes were probably specific for CNC tumours.

DISCUSSION

In this study, CGH showed a low level amplification of 2p as the most consistent abnormality in CNC tumours. One tumour showed amplification of 2p16-23 narrowing the region of search. Previous genetic mapping also narrowed the affected region to an approximately 2 Mb long area between markers D2S2153 and D2S1352. Therefore, we examined the region for cytogenetic changes using FISH analysis of CNC tumours with the previously identified, as well as new, BACs from the 2p15-p21 contig.

Overall, more abnormalities were found in touch preparations than in primary cell lines, most likely the result of overgrowth of fibroblasts in cultures. Amplification of the region represented by HSRs occurred in 60% of the samples, deletions in 32%, and amplification and deletion of 2p16 in different cell populations from the same lesion in the remaining 8%. These data are consistent with the CGH data and our previous findings of inconsistent microsatellite alterations of the 2p16 region. There have been no previous reports of consistently amplified or deleted chromosomal regions in CNC tumours.

Figure 4 Deletion of 2p16 region in CNC tumours. (A) FISH with BAC 400-P-14 (red) and α-satellite probe specific for chromosome 2 (green) to fibromyxoma cells (case 12). A metaphase spread and a nucleus, located in the lower left of the image, showed deletion of only the 2p16 region; the centromeric α-satellite probe showed the expected two signals. A third cell was normal, showing two copies of each probe. (B) Interphase FISH to ovarian tumour cells (case 29) with BACs 400-P-14 (red) and 422-A-6 (green) showed deletion of BAC400-P-14; BAC 422-A-6 showed two signals. (C) FISH with BACs 400-P-14 (red) and 514-O-11 (green) to PPNAD cells (case 10) showed deletion of both BACs; these BACs were situated very close to each other on the physical map of 2p16 region. (D) Prostate adenoma cells used as control hybridised with BACs 400-P-14 and 514-O-11 showed two copies of both probes. (E) Ovarian tumour cells (case 41) hybridised with BACs 400-P-14 and 435-I-1 that had the partial overlap with BAC400-P-14 showed deletion of both BACs in four cells; the other three cells were normal, showing two copies of each probe. (F) Hybridisation of BACs 400-P-14 (red) and 406-J-5 (green) to PPNAD cells (case 10). Cells showed deletion of BAC 400-P-14 and two copies of 406-J-5.
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Mechanisms that underlie the process of amplification are still not well known; it is generally believed to occur during the late stages of carcinogenesis. However, in our study, when amplification was detected it occurred mostly in benign tumours. This prompted us to look for a feature in the 2p16 region that would make it different from other genomic areas. In fact, 2p16 contains the relatively infrequently expressed, aphidicolin sensitive, fragile site FRA2D. Some models of amplification involve common fragile sites which occur with varying frequencies in the population. Such sites may predispose DNA to the genomic instability observed in cancer. They may or may not affect the function of a gene. An example of this is that of the fragile histidine triad (FHIT), which maps to the FRA3B site on chromosome 3 and is involved in cell cycle regulation; its abnormalities have been associated with a variety of tumours. A different and less frequent fragile site on chromosome 11 has been associated with the common breakpoint identified in chronic lymphocytic leukaemia. The difficulty we encountered in the genomic sequencing of 2p16 and its instability in tumours may be related to the presence of the FRA2D fragile site; characterisation of the latter will shed more light on its relevance to tumorigenesis.

The pattern of cytogenetic changes (amplification or deletion) on 2p16 does not seem to be associated with any clinical or histopathological features of the tumours studied. For example, among 20 PPNAD cases examined in this study, eight showed deletion, nine showed amplification, and two showed both. A remarkable observation is that the changes of the 2p16 region were observed in tumours from CNC patients, with and without PRKAR1A mutations (mapping to 17q and 2p and elsewhere, respectively). We also analysed CNC1 and CNC2 tumours with regard to their type of cytogenetic abnormality (amplification or deletion); 18 of 23 tumours from a total of 28 CNC1 tumours showed amplification and five showed deletion. From 14 of 16 CNC2 tumours, an equal number of tumours (n=7) showed amplification or deletion. This is not a statistically significant difference (p=0.07) but it may represent a trend for 2p16 amplification to be more frequent in CNC1 tumours.

Because the phenotypes of CNC1 and CNC2 patients are not significantly different, it has been suggested that both CNC genes could be involved in the same molecular pathway. This study certainly supports this notion. From our results, the following mechanism of tumorigenesis in CNC1 emerges (fig 5):
a germline mutation of PRKARIA serves as the "first hit", and LOH of PRKARIA as the second hit (although it may not always be necessary), whereas 2p alterations serve as yet another molecular event that may be necessary for tumour formation in this syndrome.

In summary, our results suggest the presence of putative candidate gene(s) on 2p16 that may be responsible for CNC2 and have oncogenic function in at least a subset of families. Our data also imply that cytogenetic alterations in the 2p16 region may be one of the earliest events in the pathogenesis of CNC tumours.

ACKNOWLEDGEMENTS

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References

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