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Investigation of Chromosome 9q22.3-q31 DNA Marker Loss in Odontogenic Keratocysts

N.J. Lench,[1,2] A.S. High,[1] A.F. Markham,[2] W.J. Hume[1] and P.A. Robinson[1,2]

[1]Division of Dental Surgery, Leeds Dental Institute, Leeds LS2 9LU; and [2]Molecular Medicine Unit, St. James's University Hospital, Leeds LS9 7TF, U.K.

Multiple basal cell carcinomas and odontogenic keratocysts of the jaws are a feature of the inherited naevoid basal cell carcinoma syndrome (NBCCS), although both occur more commonly as single, sporadic cases. The NBCCS gene has been mapped to chromosome 9q22.3-q31 and loss of heterozygosity for DNA markers from this region has been observed in familial and sporadic basal cell carcinomas. Based on these observations, we undertook a pilot study to determine if a similar pattern of chromosome loss occurs in odontogenic keratocysts. DNA extracted from microdissected odontogenic keratocyst epithelium was examined for loss of heterozygosity for six polymorphic DNA markers mapping to human chromosome 9q22.3-q31. Allelotype loss was detected in epithelium from three, single, sporadic odontogenic keratocysts. These results implicate homozygous inactivation of the NBCCS gene in the initiation and progression of the odontogenic keratocyst. Copyright © 1996 Elsevier Science Ltd

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INTRODUCTION

The developmental odontogenic keratocyst, or primordial cyst, is a common lesion of the jaws that is thought to arise from dental lamina or its remnants (glands of Serres). Odontogenic keratocysts may be small in size and single, but, more often, are large and multilocular and usually present in the retromolar regions of the mandible. The cysts can be bilateral as well as involving both jaws, are continuous in development and can become very large, causing expansion of the jaws. Unlike other cyst types, the odontogenic keratocyst can assume a locally aggressive and destructive behaviour. If left untreated, the cysts cause considerable local tissue damage and can extend into the ascending ramus. A significant clinical problem is the high recurrence rate (12–65%) observed following surgical enucleation of cysts [1, 2]. This may be due to a number of reasons: (1) proliferation of remnants of dental epithelium not associated with the original keratocyst; (2) incomplete removal of the original cyst lining; and (3) activation of the remaining dental lamina or of satellite cysts.

Odontogenic keratocysts are distinguishable histologically from other cyst types by a characteristic microscopic appearance and increased epithelial mitotic activity in suprabasal layers [3, 4]. Cysts are lined by regular keratinised stratified squamous epithelium with a well defined, often palisaded basal layer consisting of columnar or cuboidal cells. There is debate as to whether or not the odontogenic keratocyst should be regarded as a benign neoplasm since cyst epithelium apparently has a high rate of proliferation and behaves in an invasive-like manner due to active growth of the connective tissue wall [5, 6].

Odontogenic keratocysts can occur both in isolation as single, sporadic cysts or in multiple numbers as a feature of the inherited naevoid basal cell carcinoma syndrome (NBCCS) (Gorlin–Goltz syndrome) [7]. NBCCS is a complex, pleiotropic, autosomal dominant disorder associated with a spectrum of developmental abnormalities and a pre-disposition to a number of different neoplasms, in particular basal cell carcinomas (BCCs). In NBCCS, odontogenic keratocysts of the jaws often develop during the first decade of life to peak in frequency during the second or third decades. This is approximately a decade earlier than the much more common single, sporadic odontogenic keratocyst typically seen in non-NBCCS patients. It has been proposed that the underlying genetic defect in NBCCS may be a mutation in a tumour suppressor gene, as evidenced by the relatively early occurrence of multiple BCCs in NBCCS patients [8]. This is analagous to the situation in hereditary retinoblastoma where multiple tumours arise at an early age, consistent with Knudson's theory of homozygous tumour suppressor gene inactivation [9]. The process frequently occurs by point mutation of one gene homologue, followed by the loss of the second homologue by chromosomal dysjunction, deletion or meiotic recombination.

The NBCCS gene has been mapped to human chromosome 9q22.3-q31 using genetic linkage studies [8, 10]. Recent fine

Correspondence to N.J. Lench.
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genetic mapping has narrowed the region known to contain the NBCCS gene to an approximately 2.6 centiMorgan (cM) region encompassed by the DNA markers D9S196 centromeric and D9S180 telomeric [11]. Using microsatellite repeat DNA markers and restriction fragment length polymorphisms, loss of heterozygosity has also been demonstrated for this region of chromosome 9 in both sporadic (50–70%) and hereditary (50%) basal cell carcinomas, providing further evidence of the involvement of a tumour suppressor gene at this locus [8, 12]. Therefore, to determine if a similar pattern of chromosome loss is a feature of odontogenic keratocysts, we undertook a pilot study to investigate the loss of heterozygosity for six DNA markers mapping to chromosome 9q22.3-q31.

METHODS

Patients

Patient 1. A 46-year-old male with a single, sporadic, large recurrent odontogenic keratocyst. No family history or other clinical symptoms of NBCCS.

Patient 2. A 60-year-old male with a single, sporadic, odontogenic keratocyst. No family history or other clinical symptoms of NBCCS.

Patient 3. A 29-year-old female with a single, sporadic, odontogenic keratocyst. No family history or other clinical symptoms of NBCCS.

Patient 4. A 64-year-old male with recurrent odontogenic keratocysts of the right mandible. No family history or other clinical symptoms of NBCCS.

Patient 5. A 10-year-old male with large, recurrent odontogenic keratocysts in both upper and lower jaws. The patient displayed hyper-telorism with prominent supra-orbital ridges. There was no family history of NBCCS although the patient's clinical features were consistent with the diagnosis of NBCCS.

Patient 6. A 12-year-old female with recurrent odontogenic keratocysts. The patient had the characteristic NBCCS facial appearance, post-axial polydactyly and palmar and plantar pitting. The patient's father had a similar facial appearance, a history of numerous basal cell carcinomas, recurrent odontogenic keratocysts, palmar pitting and post-axial polydactyly. The patient's brother also shared the same facial appearance, and had rib and vertebral abnormalities as well as palmar and plantar pitting

Patients 1–4 were assumed non-NBCCS, patient 5 was considered a *de novo* case of NBCCS and patient 6 a familial case of NBCCS.

Tissue microdissection and DNA extraction

This procedure takes advantage of the natural tendency for odontogenic keratocyst epithelium to separate from connective tissue at the level of the basement membrane. Thirty micrometre thick wax sections were cut, floated on to glass slides, allowed to air dry at 20°C, then gently warmed under a bench-top lamp to soften the wax for micro-dissection, which was performed using a Zeiss dissecting microscope at × 10 magnification. In most cases, epithelium that had detached from underlying fibrous tissue was identified and easily collected. In other cases, dissection using a No. 11 scalpel blade (Swann-Morton, Sheffield, U.K.) was required. Epithelium was identified as a clear, colourless band overlying fibrous tissue that was light brown in colour. Only well-defined areas of sections were dissected, avoiding areas of folding or irregularity. A single cut through the wax section was placed along the line of proposed separation and the tissue of interest gently lifted from the glass and placed in a 1.5 ml microfuge tube. Four hundred microlitres of xylene was added to the tissue and mixed for 30 min at 20°C. The tissue was then pelleted by centrifugation at 10 000 ***g***/20 min and all traces of xylene removed. Four hundred microlitres of absolute ethanol was added to the tissue pellet, mixed and centrifuged at 10 000 ***g***/20 min. The ethanol was removed and the pellet dried completely. To this was added 500 µl of tissue digestion buffer containing 50 mM Tris–HCl pH 7.5, 1 mM EDTA, 0.5% Tween and proteinase K to a final concentration of 1 µg/µl and incubated at 37°C for 48 h. Proteinase K was then heat-inactivated at 95°C for 10 min. A sample of 1–5 µl (approximately 25–50 ng DNA) was used directly for PCR.

Constitutional DNA controls

Whenever possible, constitutional DNA required for loss of heterozygosity studies was obtained from buccal epithelia by mouthwash using the method described by Lench *et al.* [13]. Alternatively, DNA was extracted from paraffin-embedded non-cyst tissue obtained during surgical enucleation using the procedure described above.

Loss of heterozygosity studies

DNA (25–50 ng) isolated from odontogenic keratocyst epithelium was analysed for loss of heterozygosity for the following chromosome 9q22.3-q31 polymorphic DNA markers: D9S196, D9S287, D9S180, aldolase B2, D9S176 and D9S127 (listed in relative order from centromere to telomere as reported at the Third International Workshop on Chromosome 9 [14]). The polymerase chain reaction (PCR) was performed in 30 µl of 1 × reaction buffer containing 50 mM KCl; 10 mM Tris–HCl (pH 9.0); 1.5 mM MgCl; 0.1% Triton X-100; 200 µM each dATP, dGTP, dTTP, 20 µM dCTP; 10 picomoles of each primer; 1.0 µCi [α^{32}P dCTP] (3000 Ci/mmol; Amersham International, Amersham, U.K.) and 1.0 unit Taq. DNA polymerase (Promega, U.K.). Amplification cycles (× 35) were as follows: denaturation 96°C/20 s; annealing/20 s; extension 72°C/20 s, using the primers and annealing temperatures detailed in Table 1. Two microlitres of each PCR reaction was added to 2 µl loading buffer containing 95% deionised formamide/20 mM EDTA/0.05% Bromophenol Blue/0.05% xylene cyanol, heated to 75°C/2 min and electrophoresed in a 6% denaturing polyacrylamide gel containing 8 M urea for 3 h at 1750 V. Gels were dried under vacuum and autoradiographed for 16 h at 20°C.

RESULTS

Loss of heterozygosity for chromosome 9q22.3-q31 markers was detected in DNA isolated from epithelium from three odontogenic keratocysts (Fig. 1). All three cases were from single, sporadic keratocysts. Patient 1 showed allelotype loss at the D9S180 and aldolase B2 loci and retention of the marker D9S287 on the centromeric side. This sample was uninforma-

Table 1. Chromosome 9q22.3-q31 DNA marker oligonucleotide primer sequences and annealing temperatures used for the polymerase chain reaction

DNA marker	Primer sequence	Annealing temperature
D9S196	dCCAAAGTACTGGGATTACACCTC dGCCAATGCTTCATAACGCATTGC	65 °C
D9S287	dGACCTCATCACAGGATGCTCCTC dCTAACCACTACATTGTTCAAGGGTC	65 °C
D9S180	dGCCGAGATCGTGCCACTGCAC dGCTTTATTACAGTGGTTTGGAATCG	65 °C
Aldolase B2	dAGGGTGAACCAAGCATTCTG dACTGTACTCCAGCCTGGGTG	60 °C
D9S176	dGCTGGCTGTTGGAGAAAATTATATCC dCAAGCCAGGACCCAAGGACTTTC	65 °C
D9S127	dCCCTCAAAATTGCTGTCTAT dAGATTGATTGATACAAGGATTTG	55 °C

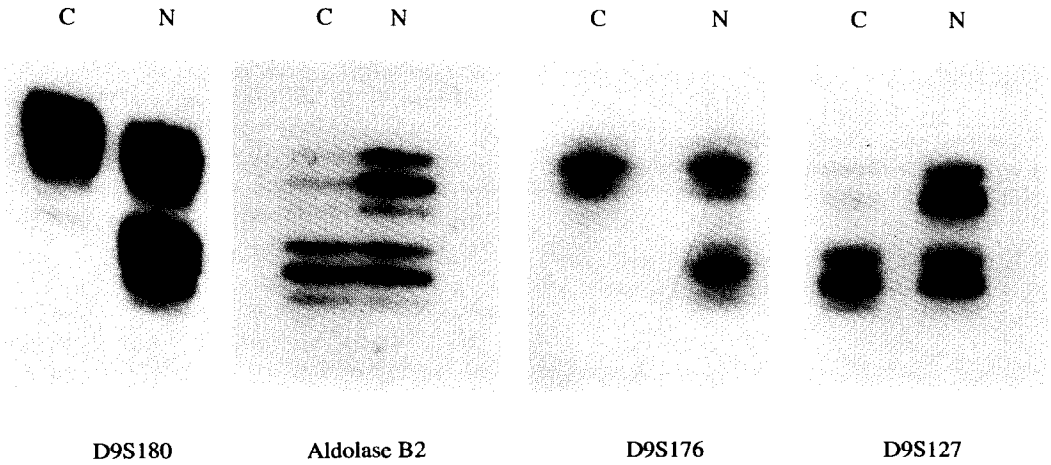

Fig. 1. Representative autoradiographs showing loss of heterozygosity of the markers D9S180, aldolase B2, D9S176 and D9S127 in DNA isolated from odontogenic keratocyst epithelium. N, normal tissue; C, odontogenic keratocyst tissue.

tive for D9S176 although the next telomeric marker tested, D9S127, was retained. Therefore, the chromosome 9q deletion breakpoints are located between D9S180 and D9S287 centromeric and aldolase B2 and D9S127 telomeric. Patient 2 demonstrated allelotype loss for markers aldolase B2 and D9S176, retention of markers D9S287 and D9S127 and was uninformative for D9S180. This places the deletion breakpoints between D9S287 and aldolase B2 centromeric and D9S176 and D9S127 telomeric. Patient 3 was uninformative for markers D9S196, D9S287 and D9S180 but showed allelotype loss for the markers aldolase B2, D9S176 and D9S127. Therefore, in this case, it was not possible to define the limits of deletion breakpoints. Loss of heterozygosity was not detected in patients 4–6 for all DNA markers tested. Combination of the data from the six odontogenic keratocysts tested define the smallest region of overlap with breakpoints between D9S287 and D9S180 centromeric and D9S176 and D9S127 telomeric (Fig. 2).

DISCUSSION

This pilot study reports the first observation of allelotype loss of chromosome 9q22.3-q31 DNA markers from odontogenic keratocyst epithelium. The smallest region of overlap defined by the observed deletion breakpoints contains the region defined by Shanley *et al.*, in loss of heterozygosity studies in basal cell carcinomas [12] as well as the naevoid basal cell carcinoma syndrome locus. This is particularly interesting, since multiple, recurrent odontogenic keratocysts of the jaws are a recognised feature of this syndrome and it has been proposed that the underlying genetic defect is a mutation in a tumour suppressor gene [8]. It is now generally accepted that detection of allelotype loss provides evidence for involvement of a tumour suppressor gene at a particular chromosomal region. Taken in combination with data obtained from loss of heterozygosity studies on basal cell carcinomas, our results strengthen the hypothesis that the NBCCS gene product functions as a tumour suppressor gene. Therefore, it is possible that the odontogenic keratocyst represents one of the first clinical manifestations of homozygous inactivation of the NBCCS gene. It is now important to extend these studies to a much larger sample size to enable an accurate estimation of the frequency of chromosome 9q loss in familial and sporadic odontogenic keratocysts.

Three odontogenic keratocysts demonstrating loss of hetero-

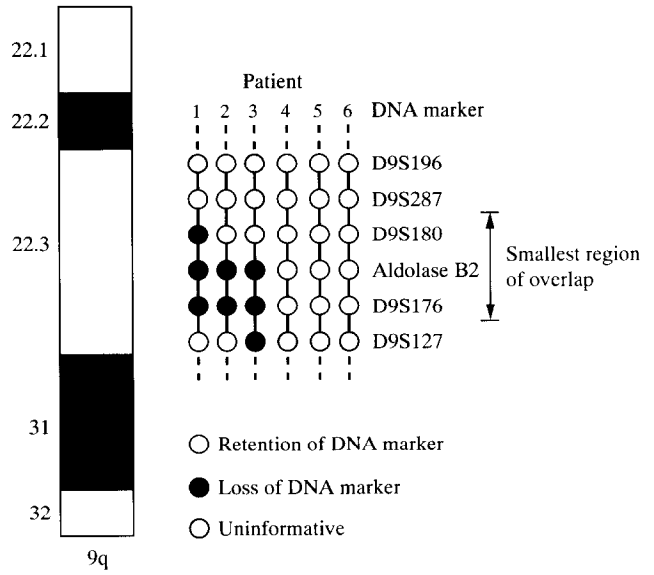

Fig. 2. Patterns of loss of heterozygosity in odontogenic keratocysts. The smallest region of overlap defined by deletion breakpoints is shown.

zygosity were single, sporadic cases. These patients did not have a family history of NBCCS or display any other clinical characteristics of the syndrome. Patient 5 showed features consistent with the diagnosis of NBCCS namely large odontogenic keratocysts of both upper and lower jaws at age 10 years and hyper-telorism, although there was no family history of the disorder. However, a high *de novo* mutation rate ($\sim 40\%$) has been reported for NBCCS, and it seems probable that this subject represents one such case [9]. Failure to detect loss of heterozygosity in the remaining cases may be due to the fact that the NBCCS gene was homozygously inactivated by two independent point mutations (not detectable by loss of heterozygosity analysis), or that a small deletion had occurred that was not detected by the DNA markers used. This could be overcome by the isolation and characterisation of novel polymorphic DNA markers from the interval defined by D9S287 and D9S176. Alternatively, the loss of an allele may have been masked by a false positive signal caused by amplification of DNA from contaminating non-epithelial tissue isolated during the microdissection procedure.

Loss of heterozygosity for this region of chromosome 9q has been demonstrated for a range of epithelial tumours, including basal cell carcinomas, squamous cell carcinomas and transitional cell carcinomas [12, 15, 16]. The observation that odontogenic keratocyst epithelium also displays loss of heterozygosity implies that the tumour suppressor gene responsible for NBCCS plays an important role in the proliferation and differentiation state of normal epithelial cells and that mutations in this gene represent an important point in the progression of epithelial neoplasia.

Numerous attempts have been made at identifying diagnostic immunochemical and histological markers for the odontogenic keratocyst [17–19]. We have shown that the epithelial-specific cell surface glycoprotein gp38, a marker of BCCs, is also strongly expressed in basal and suprabasal epithelial membranes of parakeratinised odontogenic keratocysts, and can be used to discriminate odontogenic keratocysts from other cyst types [20]. Indeed, it will be of considerable interest to see if other developmental odontogenic cysts of the jaws also display loss of heterozygosity for specific chromosomal regions.

Investigations of the growth characteristics of odontogenic keratocysts have led to the suggestion that the odontogenic keratocyst is a benign neoplasm. Scharfetter *et al.* [6] showed that the epithelium of the odontogenic keratocyst exhibited a higher rate of proliferation than the radicular cyst and that there were discrete areas of both slowly and rapidly proliferating epithelium in different parts of the keratocyst. They also demonstrated that the aggressive behaviour of the keratocyst was likely to be the result of active growth of the connective tissue wall. Ahlfors *et al.* [5] suggested that infolding of the epithelial lining into the keratocyst capsule was the result of active epithelial proliferation. Schuler and Shriver's investigation of keratin gene expression [17] suggested that there is a specific set of genetic events that give rise to the characteristic differentiation state of keratocyst epithelium. Our results strengthen the hypothesis that the odontongenic keratocyst should be regarded as a benign cystic neoplasm, since loss of heterozygosity is by definition a feature of tumorigenic tissue. This is further supported by the occurrence of multiple odontogenic keratocysts in NBCCS patients in contrast to single, sporadic cysts in non-NBCCS patients. This is consistent with Knudson's theory of homozygous tumour suppressor gene inactivation whereby in multiple tumours a pre-disposing mutation is already present in the germ line and only a single mutational event is required in a somatic cell to cause homozygous inactivation and neoplastic progression. In contrast, in sporadic tumours, two independent mutational events are required in a somatic cell. Cloning of the NBCCS gene will allow investigation of its function in normal development, its role in epithelial transformation and lead to a better understanding of the biological mechanisms of epithelial transformation and facilitate diagnosis and management of epithelial lesions.

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