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Characterization and mRNA expression in an unusual odontogenic lesion in a patient with tricho-dento-osseous syndrome

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Summary. Odontogenic lesions are rare, but can be associated with significant morbidity. While their molecular determinants are unknown, they likely express many genes common to normal odontogenesis. This study evaluated the histology and mRNA expression of an unusual odontogenic lesion in a patient with a confirmed history of tricho-dento-osseous syndrome. *Methods:* Decalcified, frozen 8 μm sections of the lesion were cut and mounted on glass slides and stained with hematoxylin/eosin for analysis. The expression of multiple genes associated with normal odontogenesis and related pathologies were evaluated by RT-PCR, where possible in samples of the hard and soft tissue components of the lesion. *Results:* Histological examination showed the lesion had large areas of irregular, dentine-like material, enamel matrix, areas of woven immature bone and multiple fully mineralised tooth crowns. Although most of the gene transcripts were amplified from both samples, some, including *DLX3/7 and Collagen I* demonstrated differential expression. *Conclusions:* This study shows the gene expression profile of aberrant odontogenesis with associated odontoma formation is similar to that of normal tooth and the genes expressed in other odontogenic lesions. While the role of altered gene expression in the development of such lesions has previously been postulated from transgenic models, this is the only report of an odontogenic lesion in a patient with TDO, and begins to elucidate possible gene interactions key to its development.

Key words: Odontogenic, Gene expression, Histology, Tricho-dento-osseous syndrome

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Introduction

The odontogenic lesions form a complex group, demonstrating a variety of histo-pathological forms with diverse clinical behaviours (Hoffman et al., 1987; Gardner, 1992; Neville et al., 1995). Although relatively uncommon, odontogenic cysts occur more frequently than true neoplasms, accounting for ~2.5% of lesions biopsied in dental offices (Phillipsen and Reichart, 2002). Sometimes tooth-like structures develop within these lesions, suggesting that they contain epithelium with inductive potential capable of recapitulating normal odontogenesis, with both processes probably regulated by similar molecular mechanisms. Although significant progress has been made in the study of the molecular interactions that control normal tooth development, our understanding of the complex molecular controls of odontogenesis remains incomplete (Thesleff et al., 1995, 1997; Thesleff and Sharpe, 1996; Maas and Ber, 1997; Peters and Balling, 1999).

Keys to understanding molecular pathways in tissue development are often found by studying pathologies where mutations of vital genes result in an altered phenotype (Satokata and Maas, 1994; Wright et al., 1994; Matzuk et al., 1995; Peters, 1998). Tricho-dento-osseous syndrome (TDO), is an autosomal dominant condition with a characteristic variably expressed phenotype with dental anomalies such as small, yellow-brown teeth with thin, hypoplastic, pitted and furrowed enamel and large, taurodont pulp chambers (Wright et al., 1997). TDO is associated with a mutation in a member of the distal-less (DLX) family of homeobox genes-DLX3 (Price et al., 1998). While *DLX3* is expressed in many different tissues, only a few show clinically discernable changes in affected individuals (Kula et al., 1996; Wright et al., 1997; Bendall et al., 2000). This is the first case report of an odontogenic lesion in an individual with this condition. The co-existence of TDO and an odontogenic lesion is of

particular interest since both involve aberrant odontogenesis.

Because odontogenic lesions occur relatively infrequently, it has been difficult to study the gene expression profile and unique molecular pathways. The most common odontogenic tumours are the ameloblastomas, derived from odontogenic epithelium (Neville et al., 1995; Melrose, 1999). A recent report describing the genetic profile of ameloblastomas using a cDNA microarray (Heikinheimo et al., 2002) showed that while many of the genes expressed were common to normal odontogenesis, there were differences. The purpose of this study was to evaluate the genes expressed in a unique odontogenic lesion and compare and contrast the results to gene expression reported for ameloblastomas and normal tooth formation.

Materials and methods

The patient was a fourteen year-old male with a positive family history (Fig. 1) consistent with the known autosomal dominant inheritance pattern of TDO (affected grandparent, parent, sibling). He presented the characteristic features of the syndrome including kinky curly hair, thin enamel and taurodontism with increased cranial bone density. Blood was collected for DNA analysis and the four base pair *DLX3* deletion associated with TDO confirmed by PCR. An oral pantograph taken as part of a routine dental examination revealed a radiolucency in the posterior right mandible associated with dental elements (Fig. 2A). A lateral oblique radiograph further revealed the bony extent of the lesion (Fig. 2B). The cyst-like lesion appeared to contain several complete tooth crowns, located adjacent to second molar tooth in the right mandibular body/ramous The lesion was exposed intra-operatively to reveal an expansive cyst of odontogenic origin expanding laterally into the buccal cortex and containing calcifications including multiple tooth crowns. A 3.0x2.0 cm mass was enucleated (Fig. 2C) and on careful dissection was found to consist of irregular fragments of white soft and hard tissue that were separated for histological and molecular studies.

Histological evaluation

A section of the lesion was decalcified in 22.5% formic acid with 10% sodium citrate for one week and processed in paraffin. The blocks were cut with a diamond microtome into 8 μm sections, mounted on glass slides and stained with hematoxylin/eosin for analysis. Van Gieson staining (saturated aqueous solution of picric acid in 1% aqueous acid fuchsin) was undertaken on additional sections to identify collagen.

Gene expression

The remaining tissue was dissected into mineralised and soft tissue components washed with Hanks buffered saline solution (HBSS) and treated with RNase/protein degrader. Cells were lysed at 45 °C for 20 minutes and the solution absorbed directly onto the Oligo (dt) cellulose. The sample was washed to remove the DNA, degraded proteins, cell debris and non-polyadenylated RNA's. Finally the mRNA was eluted and precipitated in

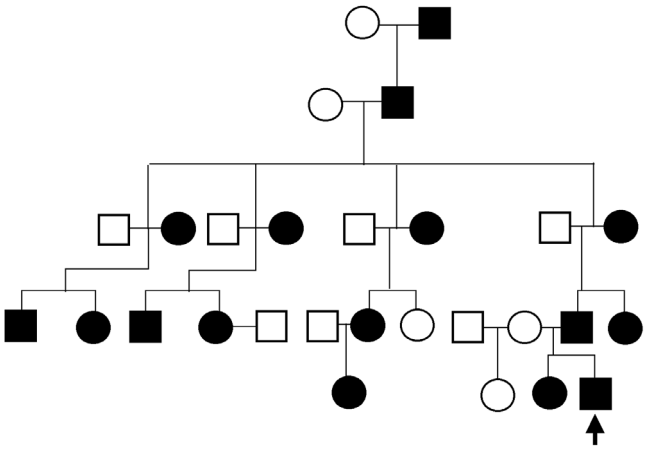

Fig. 1. Pedigree of extended family showing autosomal dominant inheritance of TDO. Patient indicated by arrow. black square: affected male. white circle: unaffected female

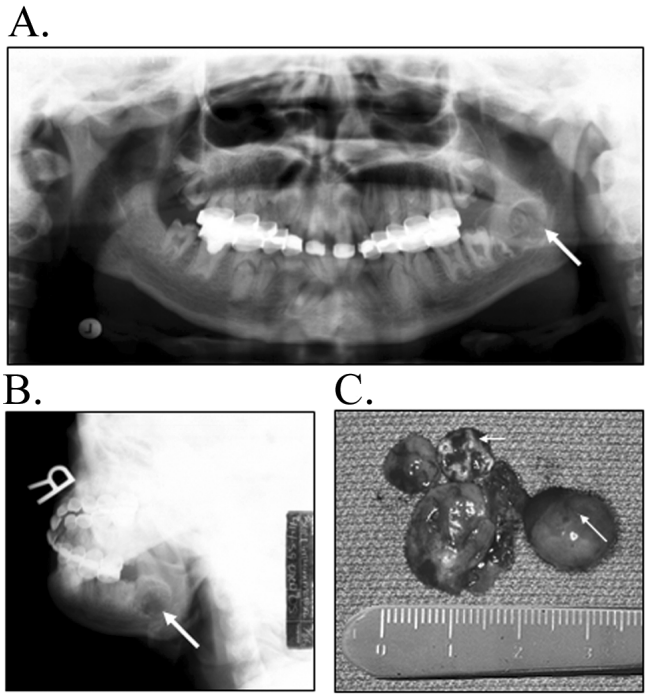

Fig. 2. Panoramic **(A)** and lateral oblique **(B)** radiographs show the well circumscribed lesion (arrows) that contains mineralizing odontogenic components. The enucleated lesion **(C)** contained multiple tooth-like structures, some with normal occlusal tooth morphology.

a mix of glycogen, sodium acetate and 100% EtOH at -80 °C. The mRNA was treated with DNase I to ensure complete removal of genomic DNA. Complementary DNA's were synthesized using a cDNA Cycle Kit™ (Invitrogen, San Diego, CA). An aliquot of the cDNA (1ml) was amplified with 1U of Taq (*Thermus aquaticus*) polymerase and GAPDH primers were added for 30 cycles of PCR to confirm integrity of the product. We were particularly interested in developing a genetic profile that included genes that had either been identified in developing teeth or in odontogenic lesions, encoding a diversity of transcription and growth factors, homeobox genes and extracellular matrix molecules. RT-PCR primers were synthesized (Table 1) based on published sequences and conditions optimised and run over 35 cycles. Experimental positive controls were either cDNAs from a human osteoblast cell line, or human placenta (known to express the genes of interest). A semi-quantitative analysis of gene expression was performed comparing expression to that of a standard (GAPDH). The products were electrophoresed on 2% agarose gels, stained with ethidium bromide and viewed and photographed under ultra-violet light. Band sizes were determined by comparison with a DNA molecular weight marker, and the appropriate amplicon verified by nucleotide sequencing.

After establishing that DLX3 transcripts were indeed expressed in the samples, additional DLX3 RT-PCR primers were designed to span the region containing the deletion found in the mutant allele sequence. The RT-PCR product (180bp) was again resolved by gel extraction, and sequenced.

Table 1. RT-PCR primer squences.

GENE	RT-PCR PRIMER SEQUENCE
BMP-2	S-TTGCGGCTGCTCAGCATGTT AS-CATCTTGCATCTCTGTTCTCGGAA
BMP-6	S-GCTAAATGCCATCTCGGTTCT AS-AACTCAGCTTGCTACAGACCA
Cadherin 11	S-CCAACAGCCCGATAAGGTATT AS-TTGGGAGCATTATCGTTGACA
Collagen I	S-CTGACCTTCCTGCGCCTGATGTTC AS-TTGGACGGTTGGTGCCCCAGAC
Collagen VIIIA	S-CCTGGGTCAGCAAGTACCTC AS-TTGTTCCCCTCGTAAACTGG
DLX3	S-ATAGAGCGGCTCCTTCGATCGC AS-GGGCTTGCCGTTCACCATGC
DLX-7	S-GGGAAGGGGTCTAAGTGTAGTAGA AS-CCCTTCCCAGATTCACCATCATCT
FOS	S-TTTATAGCTGGGCGGAAGTGG AS-CGTCCTGGACAAAGGTCACC
SHH	S-GTGGCCGAGGAAGACCCTAA AS-CAAAGCGTTCCAACTTGTCCTTA
TGFß	S-GCGTGCTAATGGTGGAAAC AS-CGGTGACATCAAAAGATAACCAC
TNFRSIA	S-TGCCTACCCCAGAGATTGAGAA AS-ATTTCCCACAAACAATGGAGTAG

Results

Histology

Histological examination revealed the lesion consisted of undifferentiated mesenchymal cells and odontogenic elements. Cystic cavities partially lined by stratified squamous epithelium contained an accumulation of enamel matrix-like products. This odontogenic epithelium supported numerous smaller coalescing areas of the amorphous matrix material (Fig. 3A,B). A small area of apparent mild epithelial dysplasia was noted on one of the sections of cyst lining. Adjacent to the epithelium were large areas of aberrant dentine containing irregular tubules (Fig. 3C). There were multiple fully mineralised tooth crowns within the cystic areas, suggesting the concomitant development of a compound odontoma. It was therefore concluded that since the lesion did not easily fit into the standard 1992 World Health Organization (WHO) Classification of Odontogenic Tumours groups (Kramer et al., 1993; Phillipsen and Reichart, 2002) it was diagnosed as aberrant odontogenesis with associated odontoma formation.

Van Gieson staining identified collagen in the mineralised dentine and osteoid components as well as in the enamel matrix, but failed to confirm its presence in the large areas of undifferentiated mesenchymal cells of dental follicular origin (Fig. 3D).

Gene expression

The tissue was separated initially into soft and hard elements, and gene expression analysed for both samples. However, due to the relatively acellular nature of the mineralised sample, and the resultant difficulty in extracting sufficient RNA for analysis, some gene expression studies were only performed on the soft tissue component. Results were confirmed by performing the analyses in duplicate. A summary of the gene expression is shown in Table 2. Although some of

Table 2. Gene expression in soft and mineralized tisse samples.

GENE AND FUNCTION	EXPRESSED IN Min/Soft
BMP2-Growth Factor	Y/Y
BMP6-Growth Factor	Y/Y
Cadherin 11-cell Adhesion Receptor	-/Y
Collagen I-ECM	Y/N
Collagen VIIIA-ECM	-/Y
DLX3-Homeobox Transcription FActor	Y/N
DLX7-Homeobox Transcription Factor	Y/Y
FOS-Proto-oncogene	-/Y
SHH-Epithelial(Mesenchymal Signal	-/Y
TGFß-Growth Factor	-/Y
TNFRSIA-Tumor Necrosis Factor Receptor	-/Y

the gene transcripts were expressed in both samples, others such as *DLX3, DLX7 and Collagen I*, were differentially expressed. *Collagen I* was expressed in the mineralised tissue but not in the soft tissue in concurrence with the Van Gieson staining. Although expressed in both tissues, DLX3 was expressed at higher levels in the mineralised tissue samples, and DLX7 appeared to be expressed at higher levels in the soft tissue relative to GAPDH (Fig. 4). Further analysis determined that the mRNA transcripts expressed in both samples encoded the mutated *DLX3* characterized by the four-nucleotide deletion. Three genes that were highly expressed in ameloblastomas – the oncogene, cFOS, the tumour necrosis factor receptor TNFRS1A, and Collagen VIIIA, were also expressed in the lesion (soft tissue only analysed and agarose gel not shown). Genes associated with normal odontogenesis that were markedly down regulated in ameloblastomas (Heikinheimo et al., 2002), but implicated in other diseases of the head and neck such as sonic hedgehog (SHH) and transforming growth factor beta (TGF-ß1) were also expressed in the lesion.

Discussion

The complex signal transduction mechanisms regulating normal odontogenesis are only beginning to be understood. While the molecular mechanisms that initiate and promote development of odontogenic tumours are likely similar to those operating during normal odontogenesis, there clearly must be fundamental differences (Thesleff and Sharpe, 1997). This study verified expression of multiple transcription factors, growth factors and extracellular matrix proteins that are also expressed during tooth development and in the development of other odontogenic tumours (Heikinheimo et al., 2002). The lesion described here has a markedly different phenotype to that of the follicular ameloblastoma however despite the differences we did not identify major differences in the expression of known odontogenic tumour associated genes between the two types of lesions. Expression of TNFRS1A, cFOS and Collagen VIIIA in both ameloblastomas and in aberrant odontogenesis suggests that although potentially important, they are unlikely to be the key molecular determinants of neoplastic change and growth critical for lesion phenotype determination. However, since both cFOS and TNFRS1A are involved in the induction of activator protein-1 (AP-1) activity that is itself associated with cell proliferation, cell differentiation, and apoptosis, in addition to oncogenic transformation, they are likely important in growth. Cadherin 11 (CDH 11 or Osteoblast cadherin) is thought to be involved in cell-to-cell communication in osteoblasts, shows down-regulation of expression in ameloblastomas. We found that it remained strongly

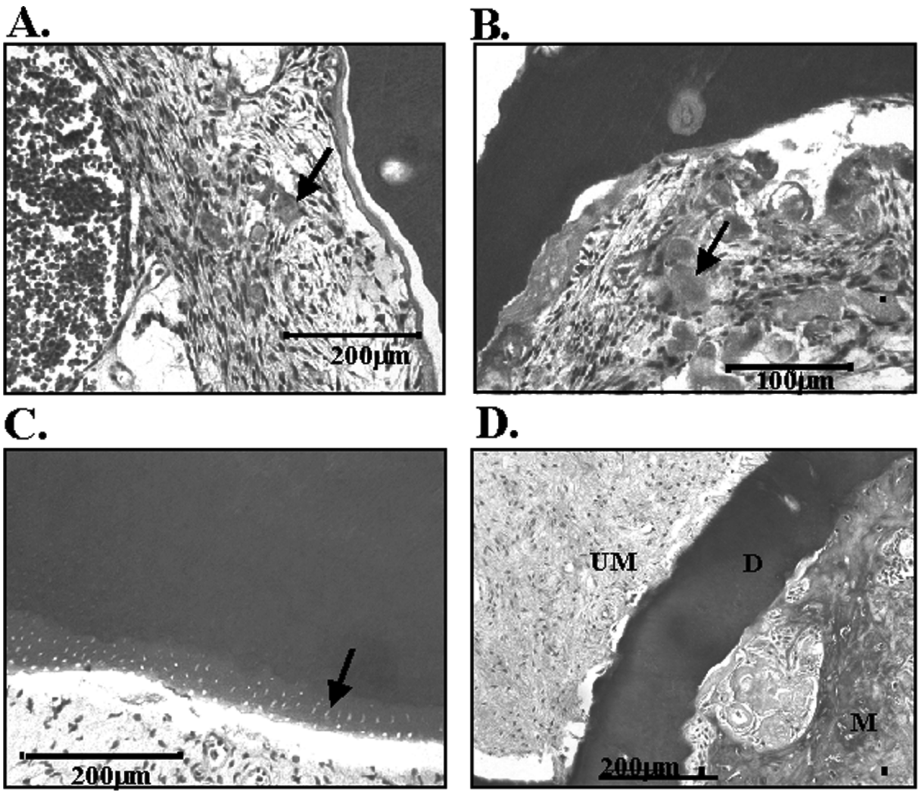

Fig. 3. Hemotoxylin and eosin stained sections of epithelium from the lesion showing accumulation of basophilic enamel matrix **(A, B)**, dentine with irregular tubules **(C)** and **(D)** Van Gieson stain indicating presence of collagen in dentine and matrix but not associated with undifferentiated mesenchymal cells.

expressed in the lesion described. It has been proposed that in the ameloblastoma, down-regulation of CDH11 accounts for the locally aggressive growth potential. The relatively high expression levels of CDH 11 found in the lesion, which does not generally display this type of growth potential, would support this hypothesis. Collagen I was expressed in the mineralised tissue but not in the soft tissue by either immuno-histostaining or RT-PCR. This was probably because the latter tissue consisted mainly of undifferentiated mesenchymal cells not yet capable of synthesizing a collagenous extra-cellular matrix.

DLX and *BMP* gene transcripts have been identified in normal odontogenesis. Although BMP proteins can mimic the effect of the dental epithelium on mesenchyme, the role of the *DLX* genes in tooth development is less clearly understood. Because this lesion developed in an individual with a confirmed *DLX3* mutation it was important to determine if there was transcription of the mutant *DLX3* allele. Expression of the normal and mutant normal and mutant *DLX3* alleles was observed. While the mutant *DLX3* is associated with thin enamel formation and large pulp chambers it is not clear how it affected growth and development in the lesion reported here. It is also possible that expression of the mutant allele contributes to the bone deposition and mineralization observed in aberrant odontogenesis.

The regulatory pathways and downstream targets of *DLX* genes have not been well characterised, although it has been suggested that like *DLX5*, *DLX3* functions as a transcriptional activator (Feledy et al., 1999; Beanan and Sargent, 2000). The temporal co-expression of homeobox genes and growth factors such as the BMPs has lead to the postulation that these molecules function cooperatively in odontogenic signalling pathways (Peters and Balling, 1999). Molecular interactions between these gene families has also been reported (Pera et al., 1998) and BMP expression identified in eight different types of odontogenic tumours including ameloblastoma, dentinoma and compound odontomas (Gao et al., 1997). However, the expression of the *DLX* genes in odontogenic lesions has not been reported until now. Here we also demonstrate the co-expression of BMPs 2 and 6 and DLX3 and 7 in aberrant odontogenesis with associated odontoma. Tissue co-expression of mRNA transcripts does not confirm specific gene interactions, however, it does provide a first level of evidence for possible involvement. Cells that express DLX3 transcripts that have been cultured from the lesion will be used in the future for studying these possible interactions.

While the role of altered gene expression in the development of odontogenic lesions has been postulated previously from murine transgenic models (e.g. Ras) (Wright et al., 1995), this is the first study to evaluate expression in this form of aberrant odontogenesis and speculate on possible gene interactions that direct it's

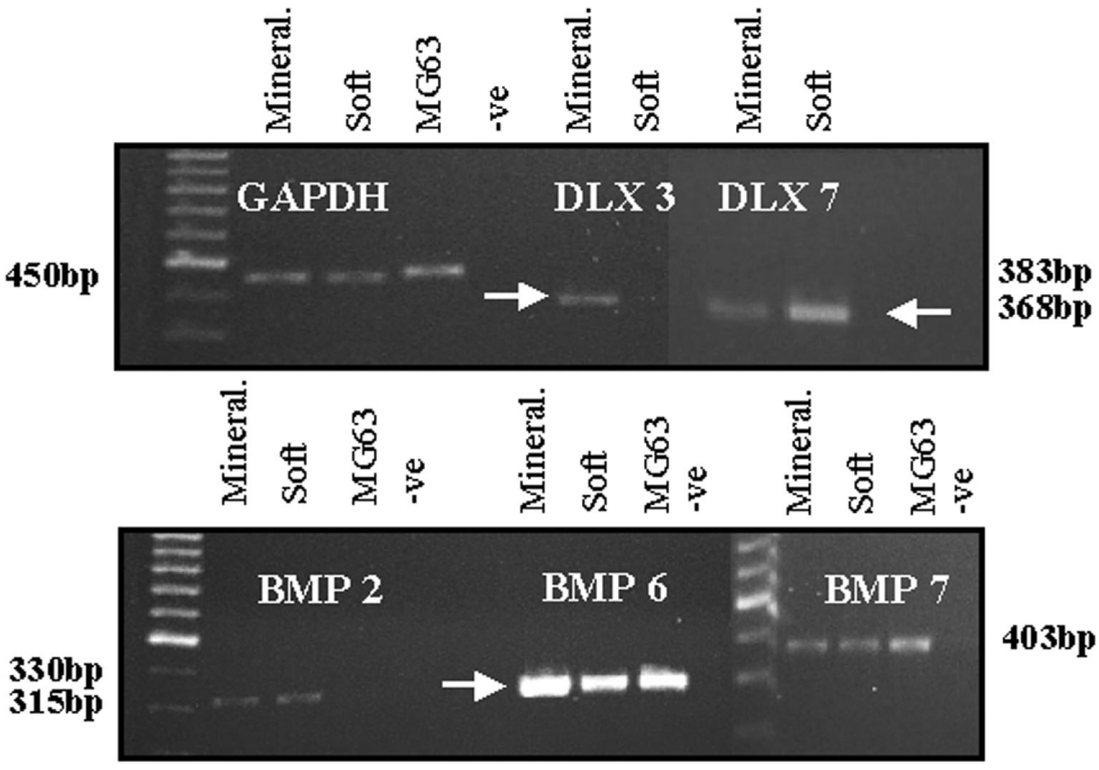

Fig. 4 . mRNA expression of DLX3/7, BMP2,6 and 7, (standardized to GAPDH) in mineralized and soft tissue components of the lesion.

development. The gene expression profiles of developing teeth, ameloblastomas and this odontogenic lesion with associated odontoma appear to be similar in many respects. However, differences clearly exist between the hard and soft tissue elements of the latter that are likely to be very important in determining the lesion phenotype. Known similarities in the molecular controls that govern the development of odontogenic lesions and those of normal odontogenesis confirm that these lesions can serve as a useful model for the study of normal tooth development. Ultimately, by characterizing the subtle molecular differences that must exist between the pathways of the different odontogenic lesions and teeth, we will provide insight into the molecular mechanisms controlling tumourogenesis with potential ramifications well beyond the field of odontogenic pathology.

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