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ARTICLE

Msx and Dlx Homeogene Expression in Epithelial Odontogenic Tumors

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SUMMARY Epithelial odontogenic tumors are rare jaw pathologies that raise clinical diagnosis and prognosis dilemmas notably between ameloblastomas and clear cell odontogenic carcinomas (CCOCs). In line with previous studies, the molecular determinants of tooth development—amelogenin, Msx1, Msx2, Dlx2, Dlx3, Bmp2, and Bmp4—were analyzed by RT-PCR, ISH, and immunolabeling in 12 recurrent ameloblastomas and in one case of CCOC. Although *Msx1* expression imitates normal cell differentiation in these tumors, other genes showed a distinct pattern depending on the type of tumor and the tissue involved. In benign ameloblastomas, ISH localized Dlx3 transcripts and inconstantly detected Msx2 transcripts in epithelial cells. In the CCOC, ISH established a lack of both Dlx3 and Msx2 transcripts but allowed identification of the antisense transcript of Msx1, which imitates the same scheme of distribution between mesenchyme and epithelium as in the cup stage of tooth development. Furthermore, while exploring the expression pattern of signal molecules by RT-PCR, *Bmp2* was shown to be completely inactivated in the CCOC and irregularly noticeable in ameloblastomas. *Bmp4* was always expressed in all the tumors. Based on the established roles of Msx and Dlx transcription factors in dental cell fates, these data suggest that their altered expression is a proposed trail to explain the genesis and/or the progression of odontogenic tumors. **(J Histochem Cytochem 57:69–78, 2009)**

KEY WORDS
epithelial odontogenic tumors
Dlx
Msx
antisense transcript
RT-PCR
in situ hybridization

ODONTOGENIC TUMORS are rare jaw pathologies (Kramer et al. 1992). Although they are habitually not accompanied by malignancy, they raise clinical problems such as (a) a regional invasiveness that may require entire maxilla removal and therefore a complex reconstruction, (b) a high recurrence rate implicating reiterative surgeries, (c) an eventual transformation into malignant tumors, and (d) very rarely, kidney and lung metastasis (Akrish et al. 2007). They constitute a heterogenous group of epithelial, mesenchymal, and mixed tumors.

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They are classified according to their histological type, anatomical site, and degree of malignancy (Akrish et al. 2007), together with the morphological similarity of tumor cells with the different stages of dental development (bud, cap, bell) and cell differentiation (Pindborg et al. 1972). Although some of them are easily diagnosed, such as the mixed odontogenic tumors (Papagerakis et al. 1999), it is often difficult to establish an accurate diagnosis for epithelial tumors (Carlson and Marx 2006; Pippi 2006). An inappropriate treatment can result from this difficulty to discriminate ameloblastomas from clear cell odontogenic carcinomas (CCOCs). Despite the accumulation of meticulous morphological and clinical observations over the years, more accurate tools, based on gene expression, are needed to discriminate between these rare heterogeneous entities (Lim et al. 2006; Hall et al. 2007).

Genetic mutations, classically reported in cancer cells, such as K-ras and β-catenin, are rare in odontogenic

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tumors (Sekine et al. 2003; Kumamoto et al. 2004; Miyake et al. 2006). Somatic mutations were identified in the enamel-related ameloblastin gene (Toyosawa et al. 2000; Perdigao et al. 2004). Animal models have shown a tight correlation between knockout expression of ameloblastin and odontogenic tumors (Fukumoto et al. 2004). Similarly, the expression of a mutated version of another enamel-related gene in amelogenin gene knockout background mice led to the development of epithelial odontogenic tumors (Gibson et al. 2007).

Few studies have been devoted to differentiate molecular expression patterns in benign and malignant odontogenic tumors. They have pointed out abnormal patterns for apoptotic molecules, suggesting an abnormal turnover of tumor cells (Kumamoto and Ooya 1999). In addition, a cDNA microarray identified a set of repressed genes in malignant odontogenic tumors compared with benign ameloblastomas (Carinci et al. 2003a). Among them, transcription factors seemed to be involved in processes underlying malignancy (Carinci et al. 2003b). These include factors playing a key role in positioning and controlling the shape of teeth during normal development.

Msx and Dlx homeoproteins are encoded by transcriptional factors involved in normal tooth development and therefore their alteration is likely to lead to malignancy. Their expression is regulated by several molecular signals including bone morphogenetic proteins Bmp2 and Bmp4 (Vainio et al. 1993; Chen et al. 1996; Bei and Maas 1998). Bmp4 has the ability to induce and/or maintain the expression of *Msx1* and *Dlx2* in mesenchyme during early tooth development (Bei and Maas 1998), whereas Bmp2 induces Dlx3 expression in other cells (Park and Morasso 2002). The mammalian Msx gene family includes Msx1 and Msx2, which have been well characterized with respect to their DNA binding and transcriptional properties.

Targeted inactivation of the *Msx1* gene in transgenic mice leads to an arrest of tooth development at the bud stage (Satokata and Maas 1994). *Msx2* mutant mice display tooth and alveolar bone defects (Satokata et al. 2000; Aioub et al. 2007). Several human gene mutations and their respective related phenotypes share similarity with those observed in animal models (Vastardis et al. 1996; Price et al. 1998; Van den Boogaard et al. 2000; Dong et al. 2005; Stephanopoulos et al. 2005).

The *Dlx* gene family is composed of six genes arranged in three clusters. During normal development, all *Dlx* genes are expressed in the dental ectomesenchyme. The *Dlx2* and *Dlx3* genes are also expressed in the epithelium (Lezot et al. 2000; Zhao et al. 2000; Ghoul-Mazgar et al. 2005). Disruption of *Dlx* gene expression induces malformed and poorly mineralized crowns in *Dlx5* mutants (Depew et al. 1999) and a lack of maxillary molars in double *Dlx1/Dlx2* mutants (Weiss et al. 1994).

The overall hypothesis merging from these studies is that odontogenic tumors are related to abnormal cell signaling. Therefore, we hypothesized that genes implied in dental-specific signals are also involved in the tumoral pathway. Because the expression levels of these genes are different in malignant odontogenic tumors and ameloblastomas (Carinci et al. 2003a), we limited our selection to transcription factors (Msx, Dlx, and Bmp) controlling odontogenesis. In our study, we also focused on recurrent tumors that (a) have an established diagnosis and (b) may differentiate to transform into malignant tumors.

Materials and Methods

The study protocol was approved by the Research Ethic Committee (Helsinki Declaration of 1975, as revised in 1983).

Tissue Preparation

Patients and Tissue Specimens. Samples were derived from recurrent odontogenic tumors of 13 patients at the department of Stomatology and Maxillofacial Surgery (Pitié Salpêtrière University Hospital). Specimens of 12 ameloblastomas and one case of CCOC are summarized in Table 1. Tumors were divided into three parts. The first part was fixed in paraformaldehyde PBS before being embedded in paraffin for pathological diagnosis according to the WHO histological typing of odontogenic tumors (Kramer et al. 1992). The second part was frozen in liquid nitrogen for RNA extraction and RT-PCR analysis. The third part was quick-frozen for ISH. Sufficient carcinoma tissues allowed fixation in Karnovsky solution (4% paraformaldehyde, 1% glutaraldehyde) for ultrastructural analysis and performance of IHC examination.

Fetal Tissue Specimen. A 9-week-old human embryonic whole orofacial tissue (EOFT), excluding brain, was frozen in liquid nitrogen for RNA extraction and RT-PCR analysis.

RT-PCR Analysis

Total RNAs were isolated from fresh-frozen tissues using TRI-REAGENT protocol according to the manufacturer's instructions (Euromedex; Strasbourg, France). RNA integrity was checked by agarose gel electrophoresis. For RT-PCR analysis, 2 μg of total RNA was reverse-transcribed with an oligo(dT) primer according to the manufacturer's instructions (Invitrogen; San Diego, CA). PCR conditions were as follows: 35 cycles of denaturation at 94C for 30 sec, annealing, and elongation at 72C for each pair of primers (Table 2). The PCR products were run onto a 2% agarose gel containing ethidium bromide.

Table 1 Clinical features and behavior of the tumors studied

Tumor	Sex	Tumor localization	Age (years)	First histological diagnosis	Age (years) and diagnosis at first recurrence	Age (years) and diagnosis at the following recurrences	Rec	Final treatment
CCOT	Female	Rt posterior maxilla	63	Ameloblastoma	64 Clear cell odontogenic carcinoma	67 Clear cell odontogenic carcinoma	2	Rt maxillectomy and radiotherapy
A1	Male	Rt posterior mandible	57	Ameloblastoma	64 Follicular and cystic ameloblastoma	–	1	Rt uninterruptive mandibulectomy
A2	Male	Rt posterior mandible	16	Plexiform ameloblastoma	42 Follicular and cystic ameloblastoma	50, 55, 62 Follicular and cystic ameloblastoma with calcifications	4	Rt mandibulectomy
A3	Male	Lt posterior mandible	36	Ameloblastoma	39 Plexiform and follicular ameloblastoma	42 Plexiform and follicular ameloblastoma 52 Plexiform ameloblastoma	3	Enucleation
A4	Male	Rt and Lt posterior mandible	45	Keratocyst and ossifying fibroma	46 Follicular and cystic ameloblastoma	49 Follicular and cystic ameloblastoma	2	Enucleation
A5	Female	Lt posterior mandible	29	Cystic ameloblastoma	33 Cystic ameloblastoma	43, 46 Cystic ameloblastoma	3	Enucleation
A6	Female	Lt posterior mandible	66	Follicular ameloblastoma	67 Follicular ameloblastoma	71 Follicular ameloblastoma	2	Enucleation
A7	Female	Rt posterior mandible	38	Keratocyst	40 Follicular ameloblastoma	–	1	Enucleation
A8	Female	Rt posterior and anterior mandible	30	Follicular and cystic ameloblastoma	–	–	0	Interruptive mandibulectomy
A9	Female	Rt posterior maxillae	24	Ameloblastoma	31 Follicular and cystic ameloblastoma	–	1	Rt maxillectomy
A10	Male	Rt posterior and anterior mandible	35	Ameloblastoma	–	–	0	Rt mandibulectomy
A11	Male	Rt posterior maxillae	19	Epidermoid carcinoma on biopsy, then Plexiform and peripheral ameloblastoma after surgery	–	–	0	Rt maxillectomy
A12	Male	Rt posterior mandible	33	Keratocyst	34 Cystic ameloblastoma	–	1	Enucleation

Rt, right; Lt, left; Rec, number of recurrency.

Preparation of Probes

The human embryonic Dlx3 PCR product (414 bp) was analyzed in a 2% agarose gel, and the amplified fragments were subcloned into bacterial expression vector, pCR2.1 (Invitrogen). In-frame cloning was confirmed by sequencing and Northern blotting of the 9-week-old embryonic whole orofacial tissues as described by Ghoul-Mazgar et al. (2005). Sense and antisense Dlx3 RNA digoxigenin-labeled probes were synthesized after linearization with BamH1 using T7 and T3 RNA polymerases, respectively. Msx1 sense and antisense RNA digoxigenin-labeled probes were synthesized from a Bluescript-SK(+) plasmid containing 350 bp of exon 2 of the mouse *Msx1* gene after linearization with BamH1 or HindIII endonuclease, using T7 and T3 RNA polymerases, respectively (Roche Diagnostics; Meylan, France).

Msx2 sense and antisense RNA digoxigenin-labeled probes (850 bp) were synthesized from pSP72 plasmid after linearization with HindIII and BglII using Sp6 and T7 RNA polymerase, respectively (Roche Diagnostics).

Amelogenin sense and antisense RNA probes were prepared from full-length RT cDNA (from W.T. Bonnass and C. Robinson, Leeds, UK), subcloned into Bluescript plasmid, and labeled with digoxigenin-UTP by in vitro transcription using T7 and/or T3 RNA polymerase (Boehringer-Mannheim; Meylan, France) used for ISH.

The Journal of Histochemistry & Cytochemistry

Table 2 Primers for RT-PCR assays

Gene	Sense primer	Antisense primer	Expective size (bp)
Msx1	5′-AAGTTCCGCCAGAAGCAGTA-3′	5′-TCAGGTGGTACATGCTGTAG-3′	328
Msx2	5′-CCTCGGTCAAGTCGGAAAATTC-3′	5′-CGTATATGGATGCTGCTTGC-3′	400
Dlx1	5′-CTACGTCAACTCGGTCAGCA-3′	5′-GGCAGAGCTAGGTACTGAGT-3′	258
Dlx2	5′-TCCTACCAGTACCAAGCCA-3′	5′-AAGCACAAGGTGGAGAAGC-3′	430
Dlx3	5′-AAGGTCCGAAAGCCGCGTA-3′	5′-CTGCTGCTGTAAGTGGGGT-3′	414
Dlx5	5′-TGGCAAACCAAAGAAAGTTC-3′	5′-AATAGAGTGTCCCGGAGG-3′	475
Dlx6	5′-GAAAACGGGGAAATCAGGTT-3′	5′-ATCATCTGTGGTCTCTGCAT-3′	420
Dlx7	5′-TAACAAGCTCCTGAAGCAG-3′	5′-ATTCACATCATCTGAGGC-3′	210
Bmp2	5′-AGGTTAGTGAATCAGAATAC-3′	5′-TCACTGAAGTCCACATACAA-3′	340
Bmp4	5′-CGAAGAACATCTGGAGAACA-3′	5′-CACTCCCTTGAGGTAACGAT-3′	420

ISH

ISH was performed as previously described (Hotton et al. 1995; Ghoul-Mazgar et al. 2005) with minor modifications: cryostat sections were hybridized with 30 μl of digoxigenin-labeled probes diluted 1:200, and the reaction was shown by antidigoxigenin Fab alkaline phosphatase–conjugated fragments (Roche Diagnostics). Histoenzymatic staining was performed for 2–18 hr depending on the tissue and the stage of development. Sections were dehydrated, mounted under a coverslip, and photographed. Tissue sections were observed and photographed on a Leica Orthoplan microscope (Leica; Solms, Germany).

IHC

Serial frozen sections (8 μm) were performed with cryostat (Bright Instrument Company; Huntington, UK). Immunolocalizations were performed with rabbit polyclonal antibodies raised against purified bovine amelogenin [gift of S. Sasaki, Tokyo, Japan, and described by Nishikawa et al. (1990)]. Sections were treated with 0.3% hydrogen peroxide in 0.1 M Tris-HCl (pH 7.6) for 10 min to inhibit endogenous peroxidase activity. After being rinsed with the Tris-HCl solution, sections were incubated overnight at 4C in Tris-HCl containing 1:30 non-immune goat serum (Nordic; Tilburg, The Netherlands) to block nonspecific binding sites. The primary antibody was applied at a 1:1000 dilution for 1 hr at room temperature. Sections were rinsed with 1% BSA in Tris-HCl and incubated with biotinylated anti-rabbit secondary antibodies (Sigma; La Verpillière, France) at a 1:200 dilution for 30 min. After incubation in 1:300 diluted-peroxydase (Sigma) for 30 min, the immunoreactive sites were visualized by the oxidization of tetrachloride 3-3′ diaminobenzidine (Sigma) 0.5 mg/ml in Tris-HCl by adding 0.03% H_2O_2 to the solution. Sections were lightly counterstained with Harris hematoxylin stain (Sigma). Sections were washed in distilled water, dehydrated, and mounted in DePex (Gurr; Osi, France). Irrelevant rabbit antibodies (Sigma) from 1:25 to 1:100 were used as negative controls replacing the primary antibodies.

Transmission Electron Microscopy (TEM)

The CCOC specimen was fixed in Karnovsky solution (4% paraformaldehyde, 1% glutaraldehyde) for 1 hr. After several washes in sodium cacodylate buffer (pH 7.4), the specimen was postfixed for 1 hr in osmium tetroxide diluted in 0.2 M sodium cacodylate buffer. The specimen was dehydrated in graded series of ethanol and left overnight in a mixture of absolute ethanol and epon 1:1. The following day, the specimen was embedded in Epon-Araldite and incubated at 60C for 1 day. Semi-thin sections were cut with a diamond knife, mounted on glass slides, stained with methylene blue (Azur II), and examined under light microscopy for orientation purposes. Ultrathin sections were obtained, collected on copper grids, and stained with 5% uranyl acetate in water for 4 min and lead citrate for 2 min. The sections were examined under a TEM Philips CM-12 (Philips; Amsterdam, The Netherlands).

Results

Histopathological Characterization of the CCOCs

The histological aspect of the recurrent carcinoma showed solid islands and strands of cells with clear cytoplasm in most areas (Figures 1A and 1B). Some tumor islands showed peripheral palisading. The tumor islands and strands were separated by mature fibrous septae. Pleomorphism and mitotic activity were occasionally observed. Periodic acid-Schiff and Alcian blue stains remained undetected.

The ultrastructural analysis of the recurrent tumor showed several cellular features: plasma membrane microvilli, numerous desmosomes, a small endoplasmic reticulum, abundant free ribosomes, glycogen rosettes, and lysosomes. Many cells showed paucity of cytoplasmic organelles with prominent vacuolization (Figures 1C–1F). Amelogenin expression was studied at the RNA (ISH) and protein (immunocytochemistry) levels. Amelogenin RNA was detected in all epithelial islands at a high magnification compared with those of stroma cells (Figure 1G). Amelogenin proteins seemed

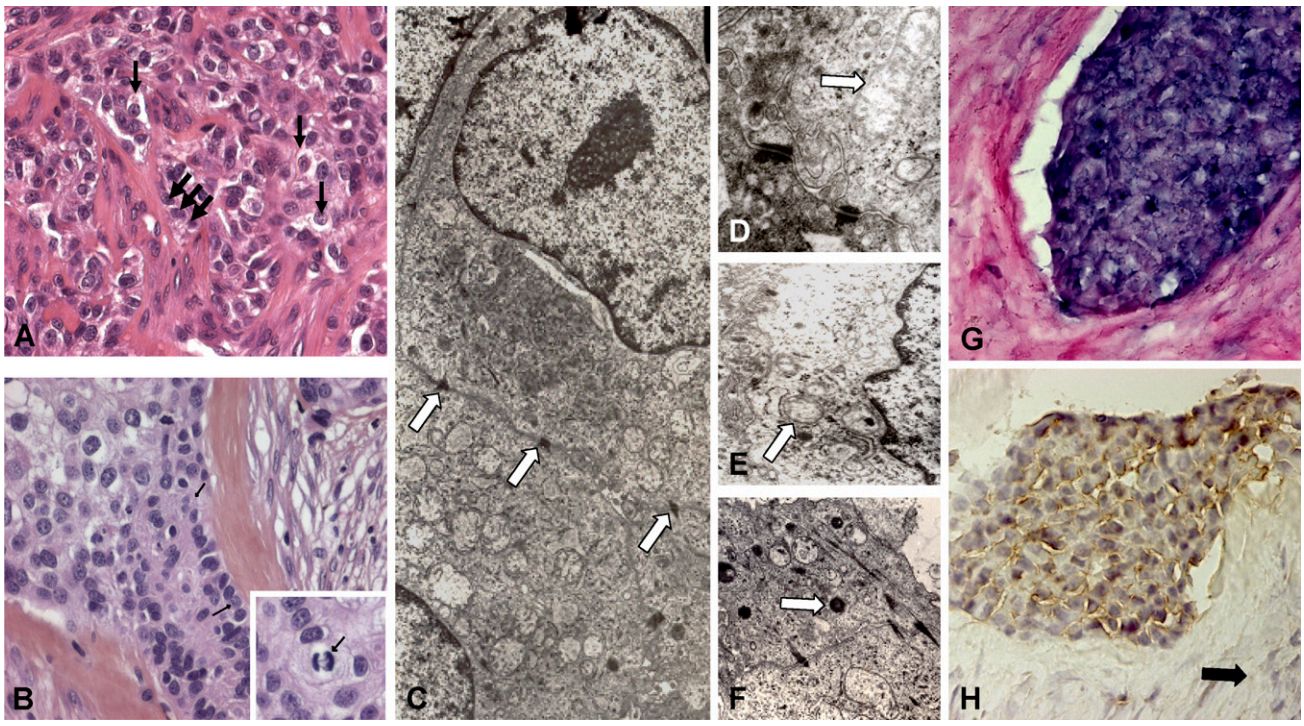

Figure 1 Structural and molecular results of the clear cell odontogenic carcinoma (CCOC). Hematoxylin–eosin–stained section of the clear cell odontogenic carcinoma shows the presence of epithelial nests with peripheral palisade cubical cells (**A,** black thick arrows) surrounding small compact interfaces. The presence of clear cells (**B,** black thin arrows) with atypia and mitosis, associated with hyalinized stroma containing giant cells, makes the diagnosis of CCOC more evident. Islands and strands of the clear cells are supported by a fibrous connective tissue stroma (**B**). Higher magnification of tumor cells shows a clear cytoplasm with uniform vesicular nuclei. (Inset: mitosis.) Desmosomes (**C,** arrows), endoplasmic reticulum, free ribosomes, glycogen rosettes, mitochondria (**D,** arrow), plasma membrane microvilli (**E,** arrow), and lysosomes (**F,** arrow) are ultrastructurally noted. Many cells exhibit a paucity of cytoplasmic organelles with prominent vacuolization. In the epithelial cells, amelogenin transcripts (**G**) and protein (**H**) are, respectively, detected by ISH and IHC. No amelogenin expression was detected in stromal cells (**H,** black arrow).

to be sequestered in the cytoplasm of epithelial cells because no labeling was noted in the extracellular stromal compartment (Figure 1H).

Msx and Dlx Homeogenes and BMP Expression Are Dysregulated in CCOCs

RT-PCR analysis failed to show significant differences in Dlx1, Dlx5, Dlx6, Dlx7, and Msx1 mRNA expression between all the epithelial odontogenic tumors and the human embryonic orofacial tissue (Figure 2). In contrast, Msx2, Dlx2, and Dlx3 mRNA remained undetectable in the unique malignant tumor (CCOC). These transcripts were always present in all the ameloblastoma samples and the orofacial embryonic tissues studied (triplicate assay).

Moreover, the RT-PCR exploration of BMP expression (Figure 3) failed to detect Bmp2 transcript in the CCOC (triplicate assay). However, this transcript was variably detected in all the other recurrent benign ameloblastomas and orofacial embryonic tissues. Comparatively, the Bmp4 transcript was regularly detected in all tissue samples studied.

Exploration of Msx2 and Dlx3 Homeogene Expression in Odontogenic Tumors

To localize Msx2 and Dlx3 homeogene–expressing cells in tumors, mRNAs of these genes were assessed by ISH (Figure 4). Different aspects were observed and summarized for three cases. Msx2 expression was inconstantly described. In fact, Msx2 transcript was detected in the epithelial cells of some ameloblastomas, as shown in Figure 4B, but only in some peripheral tumor cells, as described in Figures 4A and 4C. The Dlx3 transcript was mostly detected in all ameloblastomas, as shown in Figures 4E–4G. However, it was detected in some ameloblastomas, exclusively in the epithelial cells, as shown in Figure 4E. In other cases, it was observed in both epithelial and mesenchymal cells, as shown in Figures 4F and 4G. Neither Msx2 nor Dlx3 transcripts were detected in the clear cell odontogenic carcinoma (Figures 4D and 4H).

Msx1 antisense transcripts (Figure 5) were assessed by ISH using sense riboprobes. This method detected antisense transcripts of Msx1 in the odontogenic carcinoma. This transcript was localized in the cytoplasm

The Journal of Histochemistry & Cytochemistry

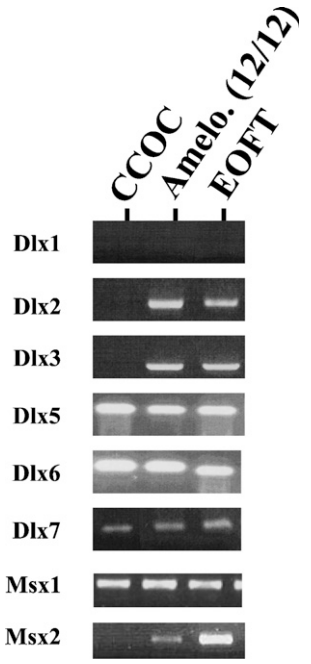

Figure 2 RT-PCR analysis of Msx and Dlx gene expression in odontogenic tumors. Msx and Dlx gene expression was analyzed in human embryonic oro-facial tissues of a 9-week-old human embryo (EOFT), 12 cases of ameloblastomas, and 1 case of CCOC by RT-PCR. Agarose gel analysis showed similar gene profile among EOFT and ameloblastomas, whereas Dlx2, Dlx3, and Msx2 remain undetected in the CCOC (triplicate essay). Dlx1 expression gene was not detected in all tissue samples.

of the epithelial (Figure 5A) and fibroblastic stroma cells (Figure 5B). However, the sense transcripts of Msx1 detected by the antisense riboprobe were only detected in the cytoplasm of fibroblastic stroma cells (Figure 5F). We did not detect signal for the sense and antisense transcripts of Dlx3 and Msx2 in this carcinoma.

Discussion

Because malignant odontogenic tumors are rare neoplasms, structural, ultractructural, and histochemical analyses were used here to confirm the diagnosis of the clear cell odontogenic tumor (Eversole et al. 1985; Kumamoto et al. 1998). Our findings therefore provide evidence that the CCOC studied here showed not only ultrastructural features (Eversole et al. 1985) but also an amelogenin expression pattern in the epithelial cells of this tumor at the RNA and protein levels as previously described (Kumamoto et al. 2001), dispelling any doubt about the histopathological diagnosis.

Although disruptions of Msx2 (Takahashi et al. 1996), Dlx3 (Roberson et al. 2001), and Dlx7 (Neufing et al. 2003; Hollington et al. 2004) homeogene expression were shown in extraoral non-dental epithelial tumors, few studies have investigated these homeogenes in odontogenic tumorigenesis.

Such a study was performed here, based on the importance of these homeogenes in the control of cell fate. At the cellular level, Msx and Dlx play diverse roles. Msx1 overexpression induces the dedifferentiation of multinuclear myotubes into myoblasts and even their transdifferentiation into another cell type such as the osteoblasts under appropriate culture conditions (Odelberg et al. 2000). Msx1 is therefore related to the maintenance of cell plasticity by inhibiting the expression of specific master genes: *MyoD* in muscular (Odelberg et al. 2000) and *Runx2* in osteo-odontogenic (Blin-Wakkach et al. 2001) cells. In the tooth germ, Msx2 was described in the enamel knot, which is considered to be a signaling center and which undergoes cell apoptosis (Vaahtokari et al. 1996). In fact, during tooth development, epithelial cells at the tip of the tooth bud stop proliferating and form the enamel knot that organizes the development of the crown shape by signal-regulated epithelial proliferation (McCollum and Sharpe 2001). Msx2 was shown to induce apoptosis in vivo (Takahashi et al. 1998), particularly during dental development (Jernvall et al. 1998) through Bmp4 proapoptotic signalization (Graham et al. 1994; Israsena and Kessler 2002). On the other hand, experimental Dlx3 overexpression in the epidermal basal cell layer is associated with premature keratinocyst differentiation (Morasso et al. 1996). It may therefore be proposed that the cell–cell communication and the growth factors that drive Msx and Dlx gene expression are essential for the control of cell fate during development, notably in the tooth germ, and may be of great interest in tumoral cell physiopathology.

Indeed, dysregulation of homeobox-containing genes is becoming increasingly recognized as an underlying

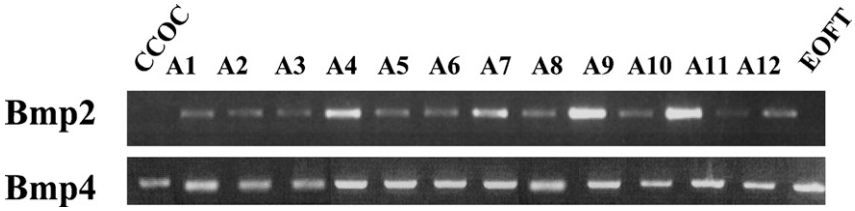

Figure 3 RT-PCR analysis of Bmp2 and Bmp4 gene expression in odontogenic tumors. Transcript expression was analyzed by RT-PCR in the CCOC, 12 cases of ameloblastomas (A1–A12), and EOFTs of a 9-week-old embryo. Agarose gel analysis showed an expected size but an irregular band intensity for Bmp2 in the ameloblastomas studied. The Bmp2 band remained undetectable in the CCOC (triplicate essay). Bmp4 transcripts seem to be regularly expressed at the expected size in all the tissue samples studied.

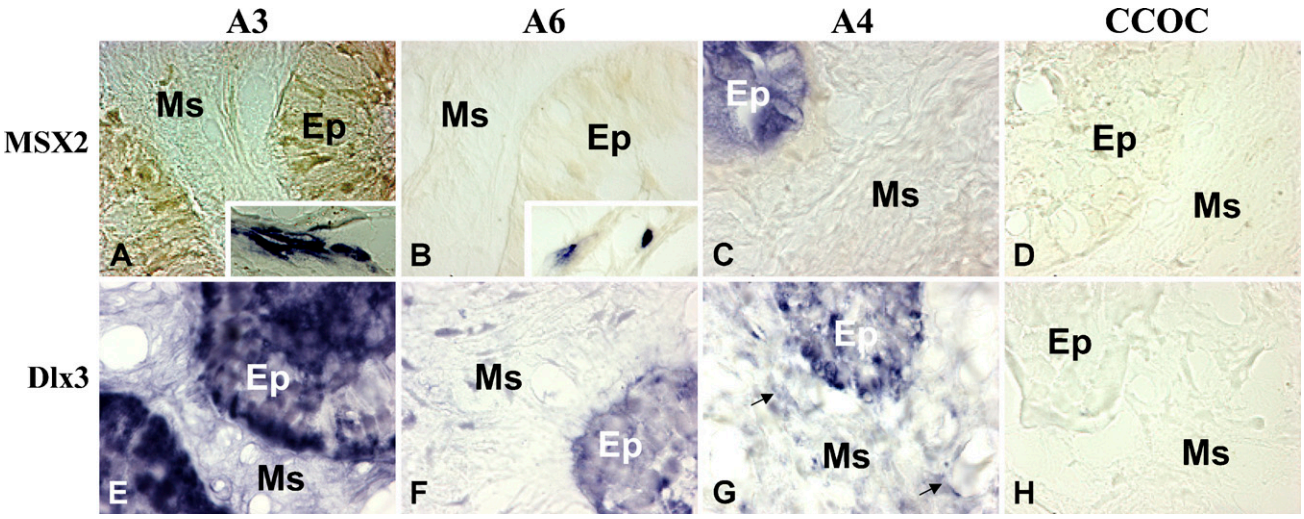

Figure 4 Detection of Msx2 and Dlx3 transcripts in odontogenic tumors by ISH. Analysis of the epithelial (Ep) and mesenchymal (Ms) compartments of the tumors showed that Msx2 transcripts are not expressed in some tumors as shown for ameloblastoma 3 (**A**), ameloblastoma 6 (**B**), and the CCOC (**D**). However, these transcripts were detected in the peripheral non-tumoral cells of ameloblastoma 3 and 6 (**A,B,** inset) and only in the epithelial compartment of ameloblastoma 4 (**C**). Concerning the Dlx3 transcript, it was always detected in the epithelial compartments of the ameloblastomas (**E–G**) but failed to be detected in the CCOC (**H**). Some ameloblastomas, as shown for ameloblastoma 4, also express the transcript in the mesenchymal part (**G,** arrow).

mechanism of tumorigenesis (Hassan et al. 2006; Shames et al. 2006; Chang et al. 2007; Takahashi et al. 2007). This fact has been described for several members of the Hox homeobox gene cluster: *HoxA9* in acute myeloid leukemia (Lawrence et al. 1996) and *HoxA13* in T-cell acute lymphoblastic leukemia (Nakamura et al. 1996; Su et al. 2006). The critical role of non-Hox homeobox genes has also been described in hematopoiesis and leukemic transformation (Owens et Hawley 2002). Precisely, the entire family of *Dlx* genes was found to be reduced in the context of the acute lymphoblastic leukemia observed in vivo and in vitro (Ferrari et al. 2003b). In other tissues, it was shown that a decrease in Dlx4 expression is associated with colorectal carcinogenesis (Hollington et al. 2004). Concerning the Msx family, Msx2 was described in various cell lines derived from human tumors, particularly in carcinoma-derived cell lines (Takahashi et al. 1996). Although Msx1 was shown to induce apoptosis in cancer cells (Park et al. 2001,2005), Msx2 was shown to exert repressive ef-

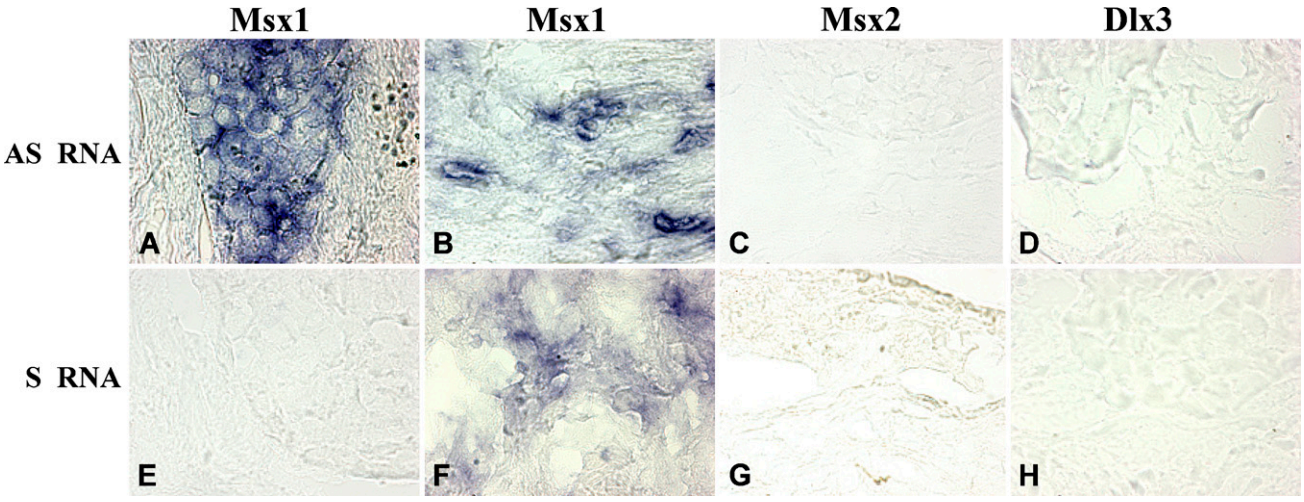

Figure 5 In situ localization of the antisense transcripts of Msx1 and the sense transcripts of Msx2 and Dlx3 in the CCOC. A specific Msx1 sense riboprobe detects antisense transcripts in the CCOC. They are localized in the cytoplasm of all the epithelial (**A**) and some of the mesenchymal (**B**) cells. No antisense transcripts were detected in this tumor using sense riboprobes of Msx2 (**C**) and Dlx3 (**D**). In contrast, specific antisense riboprobes detect the sense transcripts. In this carcinoma, no sense transcripts were detected for Msx1 either in the epithelial (**E**) or in the mesenchymal (**F**) compartments. Msx2 (**G**) and Dlx3 transcripts (**H**) were not expressed in this tumor.

fects on tumoral cells through specific induced apoptotic pathways (Hamada et al. 2005). All these data suggest the existence of the Msx and Dlx effect in tumorigenesis, albeit with potential opposite effects on cell behavior depending on the types of tumors, cells, and Msx-Dlx member.

This study provided an additional set of data on the expression patterns of Msx and Dlx transcripts in previously unexplored tumors. Benign ameloblastomas and non-tumoral tissues (EOFT) seem to have a similar profile by RT-PCR analysis. Non-tumoral tissues as odontogenic epithelium and mesenchyme in human tooth germs showed expression for the Msx1 sense or antisense transcripts. As described by ISH, the antisense RNA was exclusively observed in epithelial cells. However, it was coexpressed with sense RNA in mesenchymal cells (Blin-Wakkach et al. 2001). Thus, Msx1 sense/antisense RNA distribution in tumors imitated the physiological situation. In contrast, Msx2 ISH showed (a) an absence of expression in the stroma of benign tumors that contrasts with normal mesenchymal Msx2 expression in mouse (Aioub et al 2007) and human (Davideau et al. 1999) dental development and (b) variable expression in the epithelial cell islands depending on the tumors. Interestingly, a lack of Msx2 and Dlx expression in CCOCs was observed here. This observation of the affected Msx and Dlx homeogene expression is in line with a cDNA microarray that compared ameloblastomas with non-tumoral odontogenic tissues (Heikinheimo et al. 2002). This study showed underexpression of several transcription factors and transforming growth factor β1. It may therefore be proposed that abnormal *Msx2* gene regulation is a candidate underlying epithelial cell differentiation in ameloblastomas and CCOCs. These observations are in line with a wide spectrum of human pathologies in $Msx2^{-/-}$ mice, from eruption defects to amelogenesis imperfecta and odontogenic tumors (Suda et al. 2006; Aioub et al. 2007). Contrary to what was described during normal development, Msx2 may inhibit several apoptotic signalizations in tumoral cells (Hamada et al. 2005). Regulation of *Msx2* gene expression in some epithelial tumoral cells may be incriminated in the low or high recurrence levels (Malewski et al. 2005; Depondt et al. 2008).

Dlx2 and *Dlx3* are expressed during tooth morphogenesis (Zhao et al. 2000) and have been shown to play a key role during cell differentiation (Lezot et al. 2000; Ghoul-Mazgar et al. 2005) and apoptosis (Ferrari et al. 2003a). In addition, this study showed a lack of Dlx2, *Dlx3*, *Msx2*, and *Bmp2* expression, specifically in CCOC, compared with ameloblastomas and the normal situation.

In early tooth development, Bmp4 induces Msx1 signaling pathways (Bei and Maas 1998). Msx and Dlx homeoprotein expression is regulated by several molecular signals including bone morphogenetic proteins Bmp2 and Bmp4 (Vainio et al. 1993; Chen et al. 1996; Bei and Maas 1998; Luo et al. 2001). Bmp and their associated molecules were described in odontogenic tumors as ameloblastomas (Kumamoto and Ooya 2006). Dysregulation in Bmp signaling is suggested in our study by the evident absence of Bmp2 transcript expression in the CCOC and not of Bmp4 transcripts. Bmp2 is downregulated at the time of terminal differentiation of ameloblasts, suggesting that the differentiation process is affected in the CCOC cells. Bmp2 not only stimulates expression of Msx1 and Msx2, but it also induces Dlx2 expression (Xu et al. 2001) and transactivates Dlx3 (Park and Morasso 2002). The lack of Bmp2 may be responsible for the absence of these two homeogenes in the case of CCOC.

Epithelial odontogenic tumors are rare neoplasms arising from remnants of the odontogenic epithelium. Their pathogenesis is still unknown. To detail cell cycling perturbations in these tumors, a greater number of sample specimen is needed, but this is complicated by the disease scarcity. For the first time, we showed dysregulated *Msx* and *Dlx* gene expression between benign epithelial odontogenic tumors and one case of a rare malignant epithelial odontogenic tumor. Functional invalidation of these molecules may be explained as (a) a result of earlier disturbance events or (b) a causal event of malignant conversion. More genetic studies of these Msx and Dlx signaling molecules in odontogenic tumors should be conducted. Molecular exploration of other cases of malignant odontogenic tumors is needed to confirm our findings.

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The Journal of Histochemistry & Cytochemistry

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