EMMPRIN Modulates Migration and Deposition of TN-C in Oral Squamous Carcinoma

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Abstract. *The extracellular matrix metalloproteinase inducer (EMMPRIN), found on the surface of many tumor cells, stimulates the production of matrix metalloproteinases (MMPs) by both fibroblasts and the tumor cells themselves. To evaluate its possible role as a tumor promoter, we first overexpressed EMMPRIN, by retroviral transduction, into poorly invasive squamous cell carcinoma (SCC) cells. Secondly, we knocked down its expression using small interfering RNA (siRNA) in invasive SCC cells. The cell lines were then re-evaluated for migration on fibronectin (FN). Overexpression of EMMPRIN, promoted motility, whereas the siRNA decreased migration. The MMP expression by these variant SCC cell lines was also manipulated by EMMPRIN. The expression of MMP-2, -3, and -9 coincided with the expression of EMMPRIN. Cocultures of SCC/peritumor fibroblasts (PTF) were used to investigate tenascin-C (TN-C) matrix deposition. The cocultures overexpressing EMMPRIN, deposited several fold greater levels of TN-C compared to the control cocultures. In addition, the siRNA cocultures deposited minimal amounts of TN-C. In the presence of the broad spectrum MMP inhibitor, GM6001, TN-C deposition by the EMMPRIN overexpressing cocultures was suppressed. Thus EMMPRIN regulates migration, MMP production by SCC cells and deposition of the TN-C matrix.*

Squamous cell carcinoma (SCC) accounts for most malignancies found in the oral cavity (1, 2). The 5 year survival rate for oral SCC is approximately 50% due to invasion and metastasis (2). The extracellular matrix (ECM) regulates many aspects of cell behavior, including cell proliferation, growth, survival, migration, and invasion. It is a complex assembly of proteins and proteoglycans forming an elaborate meshwork. The precise composition varies from tissue to tissue. Tenascin-C (TN-C), a hexamer of 220-330 kDa subunits, is a large glycoprotein prominent in the extracellular matrix of embryonic tissue, tumors and healing wounds (3, 4). TN-C is a motility promoting molecule which permits and promotes cell migration and invasion (3-5). In oral cancer, the expression of TN-C first appears during dysplasia and becomes more widespread with increasing severity of the disease (3, 4).

Matrix metalloproteinases (MMPs) have been implicated in several aspects of tumor progression, including invasion through the basement membrane and the interstitial extracellular matrix (6, 7). However, the ECM is not simply a passive scaffolding, but rather a dynamic structure that is continually changing and modulates basic processes such as cell shape, movement, growth and differentiation (8, 9). MMPs alter the composition and structural organization of the ECM. It is known that MMPs are involved in ECM homeostasis and help maintain the balance between secretion and degradation of the ECM.

The SCC-associated matrix is dependent upon production of MMPs which are modulated by EMMPRIN (extracellular matrix inducer of metalloproteinases) in breast and prostate cancer (10-12). Our work has indicated that EMMPRIN expression was up-regulated in oral SCC compared with normal or dysplastic tissues (13) and is a direct modulator of cell migration. Also the expression of TN-C was increased in invasive oral SCC (3, 4). EMMPRIN expression *in vivo* correlated with that of TN-C, and we suggest that it may be an important regulator of matrix organization in oral SCC. In this study, the effect of EMMPRIN expression on several aspects of oral SCC tumor cell behavior including migration, MMP production and organization of TN-C was investigated.

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Key Words: EMMPRIN, invasion, oral SCC, TN-C, MMP.

Materials and Methods

Cell culture. The SCC9 cell line (derived from a poorly invasive tongue lesion) was obtained from Dr James Reinwald (Brigham and Woman's Hospital, Harvard School of Medicine) and has been

0250-7005/2008 $2.00+.40

described elsewhere (14). SCC9β6 (β6 "overexpression") and SCC9EMMP (EMMPRIN "overexpression") cells were generated in our laboratory by retroviral transduction with either the full length integrin β6 or EMMPRIN cDNA, respectively (15, 16). The empty vector SCC9SN cells were also established in our laboratory as described elsewhere (16). EMMPsiRNA (EMMPRIN "knockdown") and the β6C (control non-silencing RNA) cell lines were established using standard siRNA (small interfering RNA) technology (Qiagen; Valencia, CA, USA). The peritumor fibroblasts (PTF) were established by our laboratory from discarded biopsy tissue (6). The cells were routinely cultivated in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum.

Reagents. Mouse monoclonal antibodies to MMP-3 (IM36L and IM70T), and MMP-2 (IM33) were purchased from Calbiochem (San Diego, CA, USA). Monoclonal antibodies to MMP-9 (clone GE213) were purchased from Genetex (San Antonio, TX, USA). Polyclonal antibodies to actin (SC47778) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal antibody to TN-C (BC-4) was a gift from Dr. Luciano Zardi (Istituto Nazionale, Genoa, Italy). Polyclonal antibodies to EMMPRIN (34-5600) were purchased from Zymed Laboratories (South San Francisco, CA, USA).

Western Blotting. The cells were serum-starved for 24 h and then plated onto FN (10 μg/ml) for 24 h. The cells were then lysed in Nonidet P-40 lysis buffer (1. 5% Nonidet P-40, 150 mM NaCl, 0. 2% SDS, 1 mM EDTA, 20 mM Tris-HCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM Na_3VO_4, 50 mM NaF). The media were filtered through a 0. 22 μm-pore size (10 kDa cut-off) Amicon filter (Millipore; Beverly, MA, USA) and concentrated 50-fold. Protein concentration was determined by BCA Protein Assay Kit (Pierce, Rockford, IL, USA). The proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Micron Separation Inc, Westborough, MA, USA) using a semi-dry blotting apparatus (Bio-Rad, Hercules, CA, USA) as previously described (17).

The membranes were then developed by ECL (Amersham; Piscataway, NJ, USA). The blots were quantified and assigned relative value units (rvu) using an image analysis program (NIIH Image, http://www.rsb.info/nih-image). The bands quantified were from exposures that placed them within the linear range of the film.

Overexpression of EMMPRIN. The full-length EMMPRIN cDNA was overexpressed in the SCC9 cell line using the Retro-X system (Clontech, Mountain View, CA, USA). The full-length 1.6 kb cDNA of EMMPRIN was subcloned into the retroviral vector pLXSN (Dr. B. Toole, Medical University of South Carolina). The pLXSN blank vector was a negative control. The EMMPRIN construct and blank vector were transfected into the GP2-293 packaging cell line (Retro-X System, Clontech, USA) to generate the viral supernatant, which was used to infect the SCC9 cells. The expression of EMMPRIN was analyzed by Western blotting.

EMMPRIN siRNA. The siRNA duplex used in the experiment was: sense: 5'-CGGCCAUGCUGGUCUGCAAdTdT-3', antisense: 5'-UUGCAGACCAGCAUGGCCGdTdC-3'.

siRNA from Santa Cruz (sc-35298) referred to as siRNA2 was also used. EMMPRIN expression was checked by Western blot. The negative control was a non-silencing control scrambled RNA (Qiagen) that had no homology to any published mammalian gene. Transfections were performed in 24-well plates using Effectene Reagent (Qiagen) according to the manufacturer's instructions.

Wound assay. Wound assays were performed by scraping the cell monolayer with a sterile pipette tip. The plates were gently washed and recoated with 10 μg/ml fibronectin (FN) (20). Migration from the edge of the wounded monolayer was quantified by measuring the distance between the wound edges before and after injury with a computer-assisted microscope (Zeiss, Thornwood, NY, USA). The wound was referred to as "average gap area" (AGA).

Time-lapse microscopy and image processing. Conventional widefield time-lapse microscopy was performed using an Axiovert 200M-time-lapse microscope (Carl Zeiss; Thornwood, NY, USA) fitted with an Axiocam Digital Camera and enclosed in an environmental chamber (Precision Plastics, Rochester, NY) heated to 37˚C and containing 5% CO_2 in 95% air. The images were captured and assembled using AxioVision LE (Zeiss) to create movies. The AGA was determined using the AxioVision LE. Image acquisition was carried out every 10 min up to 4 h. Results are presented at time zero and at 4 h.

Cocultures. To detect ECM deposition, cocultures of peritumor fibroblasts (PTF) were grown with SCC9SN, SCC9β6, EMMPsiRNA, or SCC9EMMP cells serum-free for 24 h on tissue culture plastic. The cultures were washed, lysed and removed from the underlying matrix. The remaining ECM was scraped off the plate, solubilized, separated by SDS-PAGE and evaluated by Western blot for expression of TN-C.

Results

Differential expression of EMMPRIN. The cell lysate from the invasive and noninvasive oral SCC cells were examined for EMMPRIN expression by Western blotting. EMMPRIN levels were more than 4-fold greater in the invasive SCC9β6 cells compared to the poorly invasive SCC9SN cell line (Figure 1A).

Western blotting was also used to verify the overexpression (in SCC9EMMP cells) or "knockdown" (EMMPsiRNA cells) of EMMPRIN. EMMPRIN expression was doubled by overexpression of cDNA (Figure 1B). siRNA decreased EMMPRIN expression in the SCC9β6 cells by almost 6-fold (Figure 1C). Non-silencing RNA was used as a control (β6C cells) (Figure 1C). Identical results were obtained using an independent siRNA2 described in Materials and Methods (data not shown). Table I indicates the relative levels of EMMPRIN expression (++) and invasion (++). Note that expression of EMMPRIN correlates with invasive behavior. The expression of β1, β3, β5, and β6 integrins was unaffected by EMMPRIN siRNA or by EMMPRIN overexpression (data not shown).

Effect of EMMPRIN on SCC cell migration. Putative changes in cell behavior were evaluated by wound assay. At time zero (immediately after wounding), the AGA was 100% and as

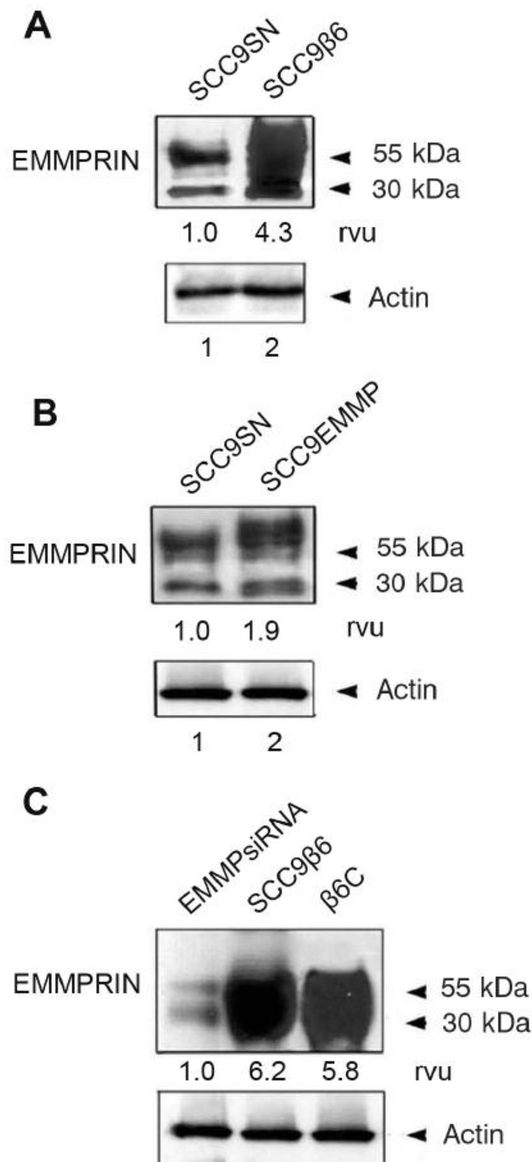

Figure 1. *Differential expression of EMMPRIN in SCC. Cells were grown as a monolayer on FN (10 μg/ml) for 24 h (serum-free), then harvested, lysed and analyzed by Western blot using antibodies to EMMPRIN. (A). SCC9SN (poorly invasive, blank vector) (lane 1) and SCC9β6 (highly invasive) (lane 2). (B). SCC9SN (lane 1), SCC9EMMP (EMMPRIN "overexpressing") cells (lane 2). (C). EMMPsiRNA (EMMPRIN "knockdown") (lane 1), SCC9β6 cells (lane 2) and the β6C cells (non-silencing, scrambled RNA) (lane 3). Relative value units (rvu) were assigned by densitometry. Note the presence of the 55 and 30 kDa form of the molecule (arrows). The membrane was stripped and probed with anti-actin antibodies as a loading control (lower panel).*

the cells migrated the AGA became smaller. By 4 h, the SCC9EMMP cells had completely closed the gap (AGA 0%), whereas the noninvasive control SCC9SN cells did not migrate (AGA 100%) (Figure 2A). In direct contrast, cell migration by the EMMPsiRNA cells was reduced (AGA 40% at 4 h) when compared to the control non-silencing RNA parental cell line, β6C (Figure 2B).

Table I. *SCC cell lines used in this study.*

Cell name	EMMPRIN	INVASION
SCC9β6	++++	+++
SCC9SN	+	+/–
SCC9EMMP	++++	+++
EMMPsiRNA	++	+
β6C	++++	+++

MMP secretion by EMMPRIN in oral SCC. The MMP-2, -3 and -9 were elevated in the SCC9EMMP cell conditioned medium (CM) compared to the SCC9SN cell CM (Figure 3 A). In contrast, the expression of MMP-2, -3 and -9 was decreased in the CM from the EMMPsiRNA cells.

Effect of EMMPRIN on the TN-C matrix. The deposition of TN-C was 4- to 6-fold greater in the cocultures of SCC9EMMP/PTF and β6C/PTF when compared to the SCC9SN/PTF and EMMPsiRNA /PTF cocultures (Figure 4A).

To determine if the increase in TN-C identified in the SCC9EMMP/PTF cocultures was MMP dependent, these cells were grown in the presence or absence of the broad spectrum MMP inhibitor GM6001. In the absence of GM6001, the cocultures deposited a rich TN-C matrix. When the cocultures were grown in the presence of GM6001, TN-C organization was virtually eliminated (Figure 4B). In addition in the presence of GM6001, the remaining TN-C was primarily the 220 kDa high molecular weight form.

Discussion

A body of literature exists with respect to stromal cell (fibroblast) production of MMPs in tumors and EMMPRIN has been shown to facilitate wound healing by inducing dermal fibroblast production of MMPs (18). In the present study, the MMP production by the tumor cell itself was specifically examined. The invasive oral SCC cells express relatively greater levels of EMMPRIN compared with noninvasive cells and EMMPRIN promoted migration on FN and appeared to autoregulate MMP production. Our laboratory previously showed that TN-C was neoexpressed in oral SCC and became a dominant feature of the SCC extracellular matrix (3, 4, 19). In the present study, the microenvironment, which was rich in TN-C, was directly modulated by EMMPRIN and MMPs. Specifically, the expression of MMP-2, -3,

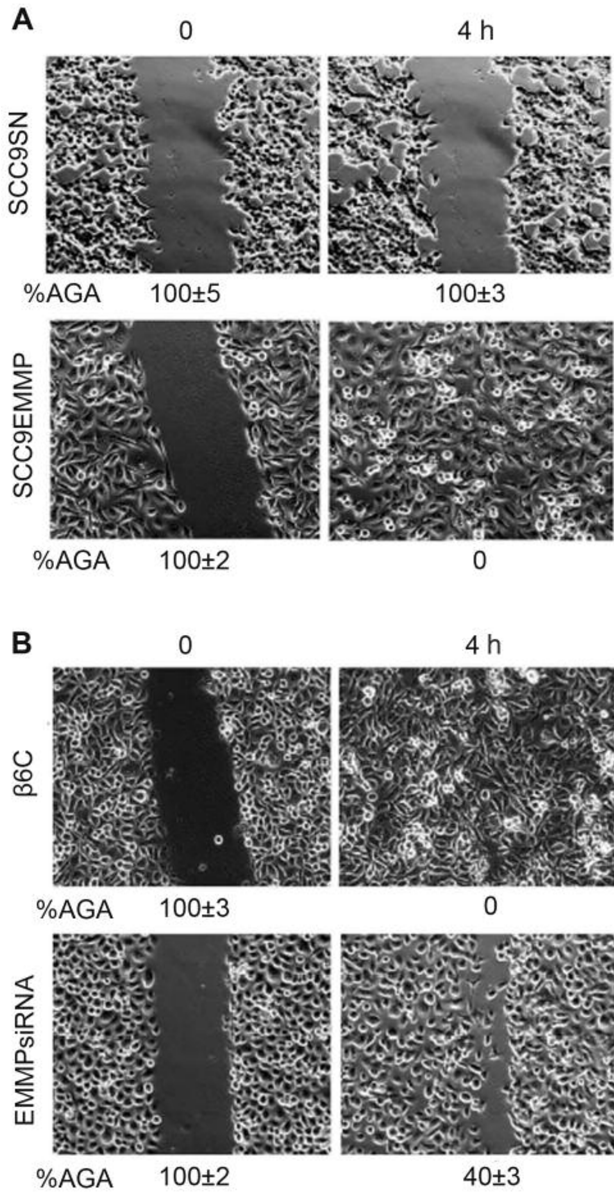

Figure 2. *Effect of EMMPRIN on SCC cell migration. Cell motility was examined by wound assay (described in Materials and Methods). (A). The SCC9SN (empty vector) cells did not move (AGA 100%) (upper panel). By 4 h, the SCC9EMMP (EMMPRIN "overexpressing") cells closed the gap (AGA 0%). (B). By 4 h, the β6C (non-silencing RNA) cells had closed the gap (%AGA 0) (upper panel), whereas the EMMPsiRNA (EMMPRIN "knockdown") had not (AGA 40%).*

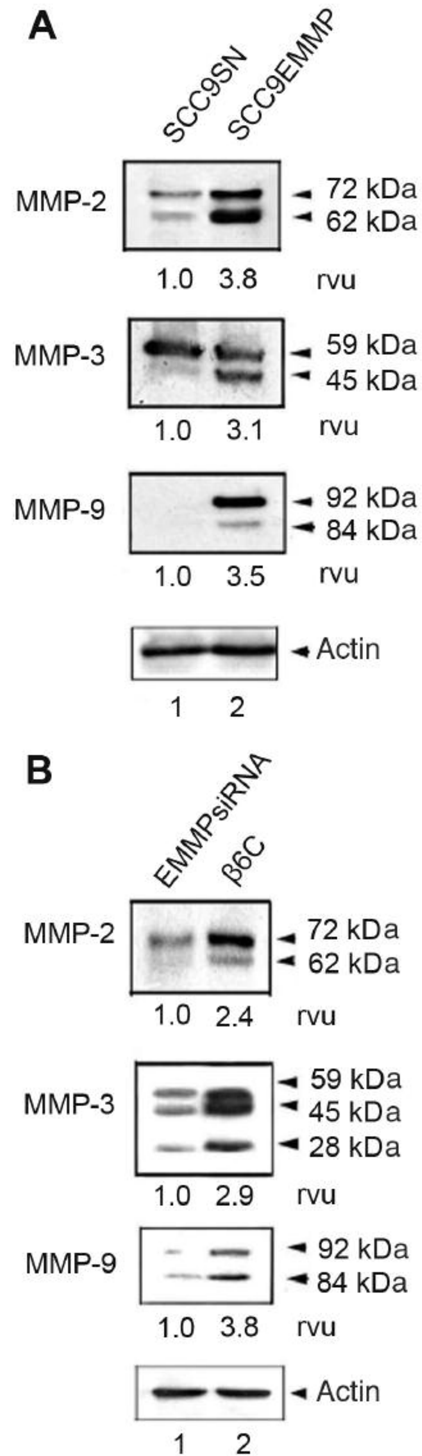

Figure 3. *MMP production by oral SCC cells. (A). SCC9SN and SCC9EMMP and (B). EMMPsiRNA and β6C cells were plated on FN (serum-free) for 24 h. Conditioned medium (CM) was removed and analyzed by Western blot for MMP expression. The major forms of MMP-2 (72 and 62 kDa), MMP-3 (59 and 45 kDa), and MMP-9 (92 and 84 kDa) are indicated by arrows and MMP representing pro and mature forms of the molecule. The blots were scanned and assigned rvu. Actin was used a loading control.*

and -9 was increased in EMMPRIN overexpressing SCC cells cultured alone or with PTF. However, this contrasted with the work of Caudroy *et al*. who found that EMMPRIN expression increased MMP-2 and -3 but not -9 in breast cancer cells (10). This difference seen between the two studies may be due to cell type specificity or general culture conditions.

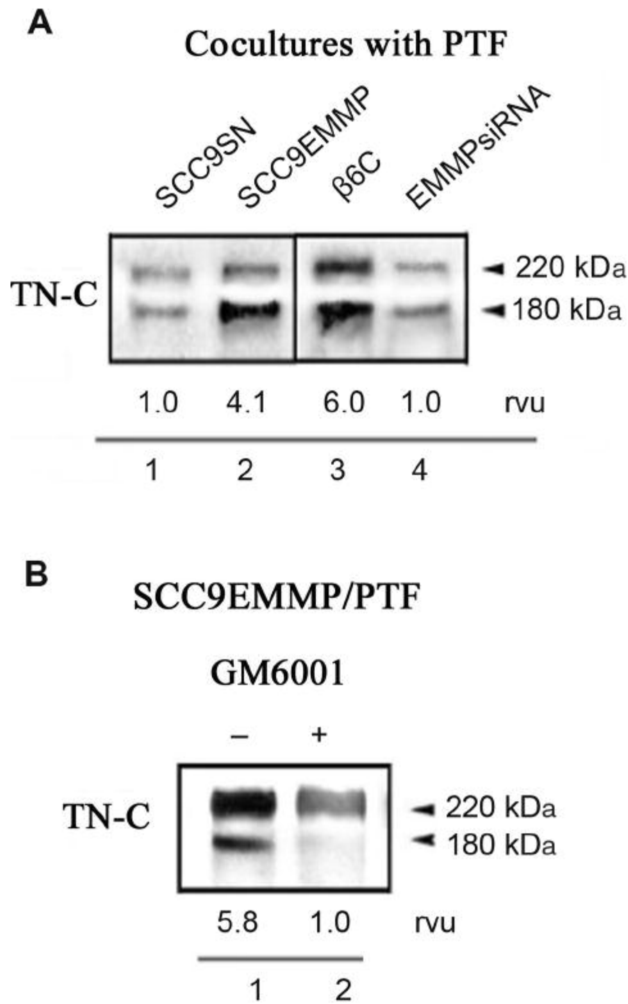

Figure 4. *TN-C deposition. (A). Peritumor fibroblast (PTF) cocultures with SCC9SN (empty vector) (lane 1), SCC9EMMP (EMMPRIN cDNA transduced) (lane 2), β6C (non-silencing RNA) (lane 3) and EMMPsiRNA (EMMPRIN "knockdown") (lane 4) cells. The matrix deposited by these cultures was harvested and assayed by Western blot for TN-C. (B). Cocultures of SCC9EMMP/PTF grown in the presence (+) or absence (–) of the broad spectrum proteinase inhibitor GM6001 for 24 h. The matrix was harvested and analyzed by Western blot for the production of TN-C. Note the 220 and 180 kDa forms of the molecule.*

We showed previously that TN-C was produced more readily in invasive cultures when compared to poorly invasive SCC (3, 4, 19), whereas in the present study, overexpression of EMMPRIN increased TN-C deposition. In contrast, suppression of EMMPRIN inhibited TN-C matrix organization. To fully investigate if TN-C deposition was dependent upon MMP production induced by EMMPRIN, the cocultures were grown in the presence or absence of the broad spectrum MMP inhibitor, GM6001. In the presence of GM6001, TN-C deposition was almost completely suppressed in the SCC9EMMP/PTF coculture.

EMMPRIN therefore seems to contribute to oral SCC growth through modulation of localized MMPs which help induce organization of TN-C. This association EMMPRIN with MMPs in tumor cells is reasonable and has been alluded to by others. It has been demonstrated that EMMPRIN can bind to MMP1 enhancing the repertoire of proteases used by the tumor cell (20). Further work needs to be done to explore the potential physical interaction between MMP-2, -3, -9 and EMMPRIN. Understanding the mechanism that mediates ECM signaling and specific matrix deposition will further contribute to the knowledge base of a wide range of diseases including developmental abnormalities and oral cancer.

Acknowledgements

This work was supported by grants from the National Institute of Craniofacial Dental Research R01 DE12856, R01 DE11930 and P01DE13904 to DMR.

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Received February 6, 2008
Revised May 7, 2008
Accepted May 14, 2008