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Evaluation of a subset of tumor suppressor gene for copy number and epigenitic changes in pleomorphic adenoma and carcinoma ex-pleomorphic adenoma carcinogenesis

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Objective. The progression of pleomorphic adenoma (PA) to carcinoma ex-pleomorphic adenoma (CXPA) encompasses several genomic alterations involving complex pathways. Tumor suppressor genes seem to play important roles in the tumorigenesis of both tumors. The aim of this study was to evaluate copy number and methylation of tumor suppressor genes' status in PA and CXPA samples.

Study Design. Eight cases of PA, 2 cases of residual PA in CXPA, and 5 cases of CXPA were studied; the latter were classified according to invasiveness and histopathological subtype. Changes in 41 tumor suppressor genes were evaluated by multiplex ligation-probe dependent amplification analysis.

Results. Copy number losses of *CASP8*, *MLH1*, and *RARB* genes were associated with PA and CXPA, while *KLK3* and *AI69125* copy number losses were exclusive to CXPA. The sarcomatoid carcinoma showed more copy number alterations compared with other subtypes. Hypermethylation of *RASSF1* was found mainly in PA and less frequently in malignant tumors.

Conclusions. *CASP8*, *MLH1*, and *RARB* tumor suppressor genes were altered by copy number losses during PA progression to CXPA. Lastly, *RASSF1* inactivation by methylation was also detected in both tumors. (Oral Surg Oral Med Oral Pathol Oral Radiol 2016;122:322-331)

Carcinoma ex-pleomorphic adenoma (CXPA) is a rare malignant tumor showing various histologic subtypes and degrees of invasiveness.[1,2] CXPA arises from pleomorphic adenoma (PA), and therefore is an interesting model for studying the progression of adenoma to carcinoma.

Tumor suppressor gene alterations, in particular promoter methylation, loss of heterozygosity, and mutations of *TP53* have been described in the pathogenesis of CXPA.[3-6] Loss of heterozygosity was found also in PA.[6] However, Gedlicka et al.[7] found no *TP53* mutations, suggesting that the malignant progression of PA is *TP53* gene independent. High *TP53* immunoexpression has been reported, but it might not be associated with sequence mutations in CXPA and PA.[6,8] Other tumor suppressor mutations have been described in the CXPA carcinogenesis process, including *P16*, *P21*, *RASSF1*, *RB*, *nm-23*, *DCC*, *hTERT*, *WT1*, and *K-ras*.[3,9-11] Several genes with putative tumor suppressor function were found in deleted regions in PA and CXPA.[12]

Aberrant methylation has been detected in a high percentage of tumors, provoking gene silencing; epigenetic changes are potentially reversible, and therefore attractive for developing new therapeutic approaches.[13] Nevertheless, few studies have examined methylation patterns in CXPA, and tumor suppressor genes involved in carcinogenesis deserve further investigation. Therefore, the objective of the present work was to determine the genomic profile of copy number and methylation alterations of a panel of tumor suppressor genes in PA and CXPA.

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Statement of Clinical Relevance

Taking into consideration that the genomic changes associated with carcinoma ex-pleomorphic adenoma and pleomorphic adenoma are not fully known, the present study aimed to identify both copy number and methylation patterns of a panel of tumor suppressor genes in these tumors.

MATERIAL AND METHODS

The present study was carried out in accordance with the ethical guidelines of our institutions (process number CEP/FOP 002/2011).

Five cases of CXPA, 8 PA, and 2 areas of residual PA in CXPA were used in this study. All 5 cases of CXPA were classified according to invasiveness (early and frankly invasive phase) and histopathological subtype.

Tumor DNA was extracted from paraffin-embedded tissue obtained with a 3-mm diameter punch and Qiagen extraction kits (Qiagen GmbH, Hilden, Germany) in accord with the manufacturer's recommendations. To improve the quality of the isolated DNA, the protocol included steps especially used for paraffin-embedded tissues, which integrate deparaffination with xylene, followed by methanol washings and 24-hour incubation in 1 mol/L sodium thiocyanate to reduce cross-links. Subsequently, the tissue pellet was dried and digested for 3 days in lysis buffer with high doses of proteinase K.

Multiplex ligation-dependent probe amplification (MLPA) is a variation of the multiplex polymerase chain reaction that permits multiple targets to be amplified with only a single primer pair.[14] MLPA was performed as described previously, using the probe mixture SALSA MS-MLPA ME001-C1 tumor suppressor probemix (MRC-Holland, Amsterdam, the Netherlands). Genes, chromosomal regions, and probe sequences are listed in Table I. This mix enabled the analysis of 41 genes that, according to the literature, may be involved in carcinogenesis.[15,16] Each probe is composed of 2 parts that hybridize to adjacent target sequences in the DNA. After the ligation step and polymerase chain reaction (PCR) amplification, each probe gives rise to a product with a unique size between 130 and 480 nt. Briefly, 100 ng DNA was denatured at 98°C for 5 minutes and hybridized with the MLPA probe mixture at 60°C for 16 hours. Ligation of the 2 parts of each probe was performed by a thermostable ligase. All probe ligation products include the same sequences and were amplified by PCR using the same primer pair at 60°C for 1 minute, 33 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute, followed by 20 minutes at 72°C, and kept cold at 4°C.

The MLPA probes for quantification of methylation are similar to normal MLPA probes for detecting the number of copies, except that the sequence detected by probe methylation was recognized by the restriction enzyme sensitive to methylation (Hhal). Twenty-four of the studied genes have a promoter region for methylation. The reaction was performed in 2 tubes; one was processed only with the hybridization reaction just described, which will provide information about changes in the number of copies. In the other tube, the hybridization reaction occurred concomitantly with the digestion of unmethylated probes, incubated with the enzyme Hhal. The probes not digested were amplified by PCR and analyzed by capillary electrophoresis.[17] All the products (copy number and methylation) were subsequently analyzed using an ABI Prism 3100 sequencer and Peak Scanner v1.0 software (Applied Biosystems, Warrington, UK).

DNA from 4 different normal salivary glands was used as a reference to calculate median values and standard deviations for every probe. Each tumor sample was analyzed at least twice. For every gene, the relative copy number was calculated by dividing the average relative peak area of the tumor by the median relative peak area of the normal reference samples. Normal DNA resulted in relative copy numbers varying between 0.85 and 1.15 for each probe. Therefore, relative copy number values, considering the standard deviation, that were lower than 0.7 were interpreted as losses, those higher than 1.3 as gains, and those 2.5 or higher as amplifications. This interpretation of MLPA data is in accord with Moerland et al.,[18] who found a 98% concordance with data obtained by fluorescence in situ hybridization spot counting, both performed on paraffin tumor material. P values $<.05$ were considered significant (Figure 1). The methylation analysis was done by comparing the peak of the unmethylated probes with methylated ones (Figure 2).

RESULTS

CXPA demonstrated 3 cases in the early phase of invasion (intracapsular <1.5 mm of invasion), and 2 cases were frankly invasive (>1.5 mm). Histologically, CXPAs were subclassified as epithelial-myoepithelial carcinoma (n = 2) (Figures 3 and 4), salivary duct carcinoma (SDC; n = 1) (Figure 3), myoepithelial carcinoma (MC; n = 1) (Figure 3), and sarcomatoid carcinoma (SC; n = 1) (Figure 4). The data of PA and CXPA cases are summarized in Table II.

Losses in copy number detected in PA samples affected the *CASP8* gene (8/8 tumors), followed by the *RARB* and *MLH1* genes (7/8 tumors). The *RASSF1* gene was methylated in 3 of 8 PA cases. Other alterations are detailed in Table III. The results in residual areas of PA in CXPAs were similar to PA; copy number losses were detected in *CASP8*, *RARB*, and *MLH1* in 1 case (1/2), and *RASSF1* was methylated in another (1/2). Details are given in Table III.

AI651963 and *KLK3* genes were identified as affected by losses only in the CXPA group (3/5), which also carried *MLH1*, *MLH3*, *CASP8*, and *PAH* losses (2/5). The copy number and methylation profile was

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