Somatic copy number alterations in pleomorphic adenoma and recurrent pleomorphic adenoma

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Objective. As the genetic changes in recurrent pleomorphic adenoma (RPA) have not yet been investigated, the aim of this study was to assess the genomic profile of somatic copy number alteration in RPA and pleomorphic adenoma (PA) by using array comparative genomic hybridization (aCGH).

Study Design. Four cases of RPA and 13 cases of PA were evaluated by using aCGH, using a 180 K platform. Data were analyzed by using Nexus Copy Number Discovery.

Results. The RPA group rarely showed any copy number alteration, except for 1 case that exhibited losses in 5 p15.33 p15.1, 5 q13.1 q35.3 and 12 q12 q13.11. The PA group also showed few copy number alterations, and the most frequent findings involved chromosomes 8: 8p21.3-p12 (gain), 8q12.1 (loss), 8p23.3-q24.3 (gain), and 8q12.1-q21.11 (gain). Genomic amplifications were revealed in the PA group, and the relevant affected genes were *MAML2* and *LIFR*.

Conclusions. PA and RPA exhibit few somatic copy number alterations and show a similar genomic profile on aCGH. (Oral Surg Oral Med Oral Pathol Oral Radiol 2020;129:59−64)

Pleomorphic adenoma (PA) is the most common type of salivary gland neoplasm. Extensive cytogenetic studies revealed that these tumors have highly specific chromosome abnormalities. The genomic findings of PAs are classified into 4 major subgroups: (1) chromosomal rearrangements involving a breakpoint on chromosome 8 (q12); (2) chromosomal rearrangements involving a breakpoint on chromosome 12 (q14-15); (3) clonal changes different from 8q12 and 12q14-15; and (4) an apparently normal karyotype, including tumors characterized by trisomy 8 or Y as mosaics.[1,2] Molecular characterization of these loci identified some important genes in carcinogenesis (*PLAG1, HMGA2*, and *CTNNB1*).[2-7]

Recurrent pleomorphic adenoma (RPA) has been associated with either increase in the number and complexity of genetic abnormalities or acquisition of promoting mutations. To date, RPA studies have focused on tumor treatment, target marker for recurrence, analysis of proliferation indices, and comparison of the stromal patterns between nonrecurrent PAs and RPAs.[8-13] So far, there has been no investigation comparing the genomic profiles of RPA and PA. The aim of this study was to assess the genomic profile of copy number alterations associated with PA and RPA through array comparative genomic hybridization (aCGH).

MATERIAL AND METHODS

Thirteen cases of PAs and 4 of RPAs were retrieved from the archives of the Pathology Department, Faculty of Medical Sciences of the State University of Campinas (Campinas, Brazil). The cases were reviewed by 2 pathologists, and the diagnoses were reviewed and confirmed according to the criteria for the histologic typing of PA in the 4th edition of the *World Health Organization Classification of Head and Neck Tumours*[2]

Tumor DNA was extracted from a 1.5-mm-diameter punch of the paraffin-embedded tissue using the Qiagen extraction kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's recommendations. To improve DNA quality, the protocol included

Statement of Clinical Relevance

This study showed that pleomorphic adenoma and recurrent pleomorphic adenoma exhibit few somatic copy number alterations and that they show similar patterns of genomic profiles by array comparative genomic hybridization. These results suggest that besides the common origin of these tumors, their tumorigenesis can be similar as well.

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deparaffinization with xylene, followed by methanol washings, and a 24-hour incubation in 1 mol/L sodium thiocyanate. Subsequently, the tissue pellet was dried and digested for 1.5 days in a lysis buffer with high proteinase K dose. The material was column purified and eluted in buffer.

Samples of tumor DNA and reference DNA (pooled from the blood of different healthy donors) were labeled by using the Enzo Genomic DNA Labeling kit (Promega, Madison, WI), according to the manufacturer's instructions. Five hundred nanograms of test DNA and 500 ng of reference DNA were cohybridized to a 180-k oligonucleotide array (SurePrint G3 Human CGH Microarray Kit 4 × 180 K design 22060; Agilent Technologies, Santa Clara, CA), according to the manufacturer's instructions. This design contains 24,011 exomic probes. Microarray images were obtained by Agilent Microarray Scanner Bundle (Agilent Technologies, Santa Clara, CA), and data were extracted by using Feature Extraction (v 9.1) (Agilent Technologies, Santa Clara, CA). aCGH data were analyzed by using the software Nexus Copy Number Discovery edition v 7.0. Genomic copy number alteration was called based on the FASST2 segmentation algorithm (significance threshold set at 5×10^{-8}) with threshold $\log_2$ ratios of 0.2 or 0.8 for gains and high-copy gains, respectively, and −0.2 or −1.0 for losses and homozygous losses, respectively.

The present study was carried out in accordance with the ethical guidelines of our institution (CEP/FCM-1155/2011:22, November, 2011).

RESULTS

Clinicopathologic and histopathologic data

The PA group consisted of 7 females and 6 males (mean age 41 years). The parotid gland was the most commonly affected site (77%), followed by the minor salivary glands (15.4%) and the submandibular gland (7.6%) (Table I). To date, none of the cases has recurred. The mean follow-up time was 46 months (range 2−316 months). The RPA group consisted of 4 females (mean age 61 years). Mean time to recurrence was 78 months (range 12−216 months) and the mean follow-up time was 113 months (range 96−137 months). In all cases, the recurrence was local and occurred in the gland affected by the primary PA (Table II).

Microscopically, PAs were observed to be encapsulated, and the neoplastic parenchyma exhibited a biphasic population composed of a mixture of luminal and myoepithelial cells. The tumor exhibited variable architecture, with formation of duct-lining structures and cell proliferation permeated by myxochondroid stroma. In contrast, RPAs showed multinodular growth, and the nodules were composed of ductal elements, epithelial and myoepithelial proliferation, and myxoid stroma. The representative morphologic features of PA and RPA are illustrated in Figure 1.

Table I. Clinicopathologic features of 13 patients diagnosed with pleomorphic adenoma

Clinicopathologic feature	*N (%)*
Gender	
Female	7 (53.8%)
Male	6 (46.2%)
Age (years)	
Mean	41 (range 24−56)
Anatomic location	
Parotid gland	10 (76%)
Submandibular gland	1 (7.7%)
Minor salivary gland	2 (15.4%)
Parapharyngeal space	1 (7.7%)
Soft palate	1 (7.7%)
Follow-up (months)	
Mean	46 (range 2−316)

aCGH data

The PA group showed few copy number alterations. The most frequent findings involved chromosome 8 and included 8p21.3-p12 (gain), 8-q12.1 (loss), 8-p23.3-q24.3 (gain), and 8-q12.1-q21.11 (gain), followed by loss at 19q13.32. The amplified genes found in the PA group were *MAML2*- 5p13.1 (1 case) and *LIFR* -11q21 (1 case) (Table III, Figure 2). The RPA group showed almost no copy number alteration, except for 1 case that exhibited losses in 5p15.33-p15.1, 5q13.1-q35.3 and 12q12-q13.11 chromosomes (Table IV, Figure 3). The only alteration found in both tumors was loss at chromosome 5p15.33-p15.31, but according to the literature, none of the genes of this chromosome is known to be related to cancer.

Table II. Clinicopathologic features of 4 patients diagnosed with recurrent pleomorphic adenoma

Clinicopathologic feature	*N (%)*
Gender	
Female	4 (100%)
Age (years)	
Mean	61 (range 50−78)
Anatomic location	
Parotid gland	4 (100%)
Time to recurrence (months)	
Case 1	216
Case 2	36
Case 3	48
Case 4	12
Follow-up (months)	
Mean	113 (range 96−137)

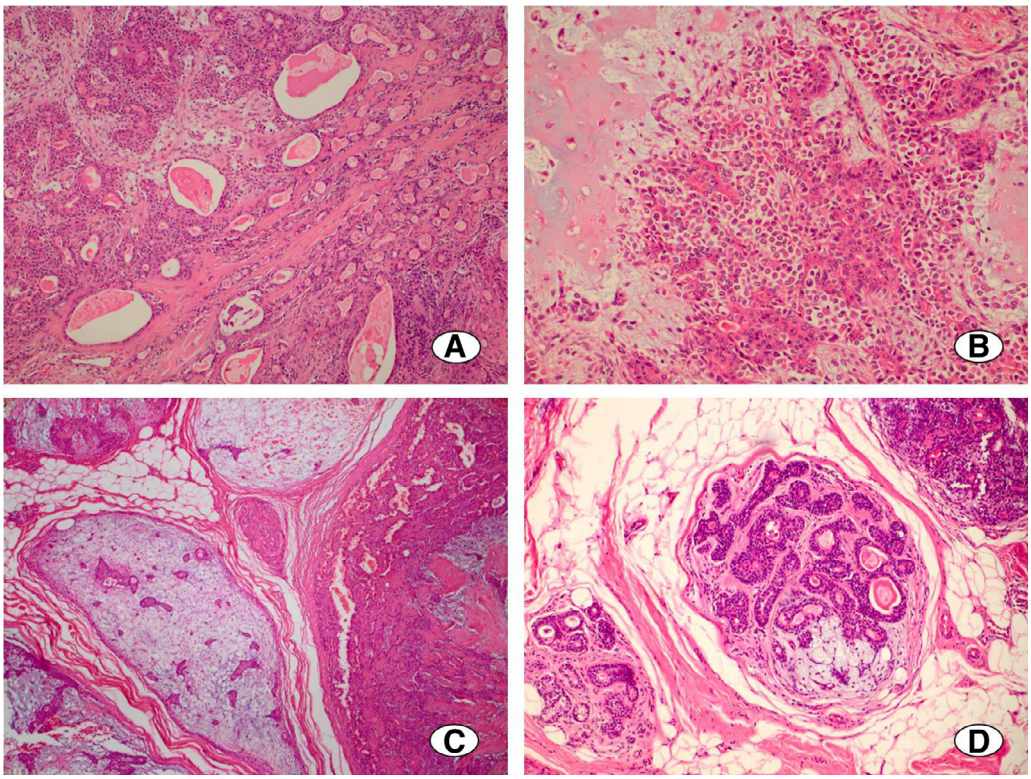

Fig. 1. **A, B,** Pleomorphic Adenoma. A well-demarcated lesion showing an admixture of epithelial, myoepithelial, and stromal components. The tumor presents a variety of growth patterns, including ductal structures and myxochondroid stroma (hematoxylin and eosin [H&E]; original magnification × 10, × 20). **C, D,** Recurrent pleomorphic adenoma showing multinodular growth. The nodules are composed of the ductal elements, epithelial and myoepithelial proliferation, and myxoid stroma (H&E; original magnification ×10, ×20).

DISCUSSION

PA accounts for about 60% of all salivary gland tumors. The reported mean age at presentation was 46 years; however, the age ranged from the 1st to the 10th decades. A female predominance was found, and the parotid gland was the most common site.[2] Important cytogenetic studies of PA have shown that 70% of the tumors are karyotypically abnormal, and the alterations

Table III. Somatic copy number alteration in pleomorphic adenoma

Case	*Loss*	*Gain*	*Amplification*
1	0	8p23.3-8q24.3 (*PCM1, WRN, WHSC1L1, FGFR1, HOOK3, TCEA1, PLAG1, CHCHD7, NCOA2, HEY1, COX6 C, EXT1, MYC, NDRG1, RECQL4*)	0
2	5p15.33-p15.31*	0	5p13.1 (*LIFR*)
3	0	0	0
4	0	0	0
5	0	12p13.2 (*ETV6*)	11q21 (*MAML2*)
6	6q16.1-q25.3 (*PRDM1, ROS1, GOPC, STL, MYB, TNFAIP3, ECT2L, EZR*)	0	0
7	0	0	0
8	8q12.1 (*PLAG1, CHCHD7*)	0	0
9	0	8p21.3-p12*	0
10	9p24.3-p21.1 (*JAK2, CD274, NFIB, MLLT3*) 19q13.32 (*ERCC2*)	0	0
11	0	0	0
12	19q13.32 (*ERCC2*)	8q12.1-q21.11 (*CHCHD7, NCOA2*)	0
13	0	0	0

*Absence of genes related to cancer.

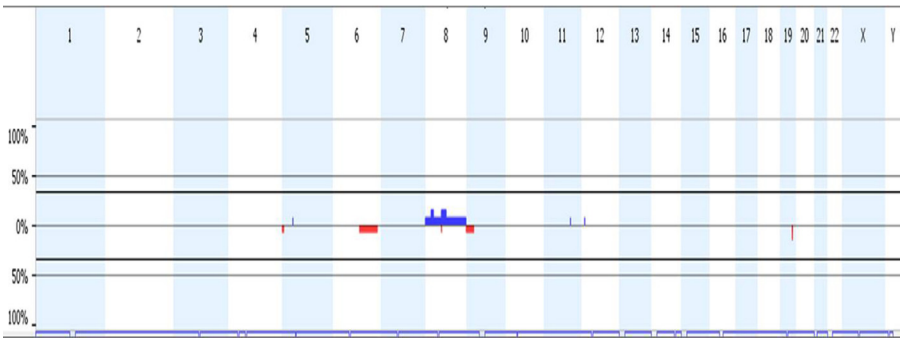

Fig. 2. Global array comparative genomic hybridization (aCGH) genomic profile exhibiting the identified copy number alterations in pleomorphic adenoma (PA) cases. The *x*-axis represents probes ordered according to their genomic position from chromosomes 1p to Xq. The *y*-axis denotes the log2 test/reference values (genomic gains in blue and losses in red are plotted above or below the 0 baseline, respectively; images adapted from the software Nexus Copy Number 7.0, Biodiscovery).

Table IV. Somatic copy number alteration in recurrent pleomorphic adenoma

Case	*Loss*	*Gain*	*Amplification*
1	0	0	0
2	0	0	0
3	5p15.33-p15.1* 5q13.1-q35.3 (*APC, PDGFRB, CD74, ITK, EBF1, RANBP17, TLX3, NPM1, NSD1*) 12q12-q13.11(*ARID2*)	0	0
4	0	0	0

*Absence of genes related to cancer.

are divided into 4 groups: (1) PAs with rearrangements at 8q12; (2) PAs with rearrangements at 12q13-15, (3) PAs with other changes different from 8q12 and 12q13-15, and (4) PAs with apparently normal karyotype.[1] The significant genes found in these rearrangements were *PLAG1* (8q12) and *HMGA2* (12q13-15).[2-7] *PLAG1* is expressed as a result of translocations that cause promoter swapping between *PLAG1* and a ubiquitously expressed translocation partner gene. *CTNNB1* and *LIFR* are the most common partners of *PLAG1*, generating the t(3;8)(p21;q12) and t(5;8)(p13;q12) translocations, respectively.[2-3,7] Cryptic gene fusions involving *PLAG1* have also been described as *CTNNB1-PLAG1* and *TCEA1-PLAG1*.[6,14]

The present study investigated the somatic copy number alteration in PA and RPA and high copy gains or amplification of the *PLAG1* was expected; however, the results showed few gains and amplification of *PLAG1*. Alterations at chromosome 8 were observed; however, only 1 case showed gain at 8p23.3-8q24.3 involving *PLAG1*. Another case displayed loss at 8q12.1. Several studies have demonstrated *PLAG1* positivity as possibly resulting from gene overexpression and not because of its amplification or high copy gain. Besides, it has been suggested that *PLAG1* expression is controlled by complex molecular mechanisms, such as translocations or by micro-RNA−mediated translational control.[6, 15,16]

HMGA2, the target gene located at 12q14-15 also showed fusions as *HMGA2-NFIB* [ins(9;12)] and *HMGA2-FHIT* [t(3;12)], but the most important event was the separation of the DNA-binding domains from potential mRNA.[4,5] No high copy gain or gain of

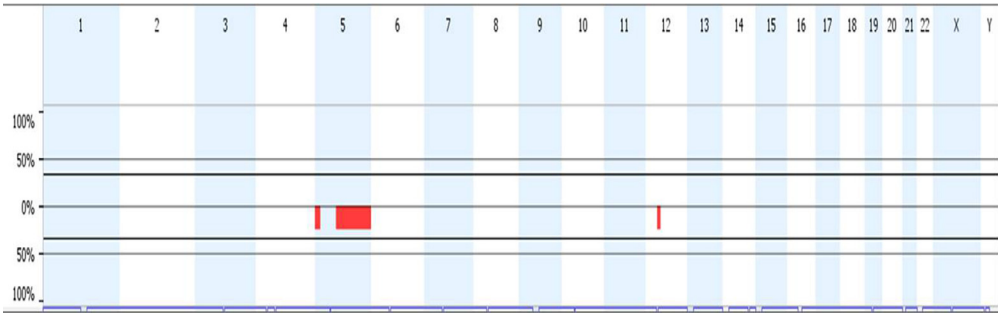

Fig. 3. Global array comparative genomic hybridization (aCGH) genomic profile exhibiting the identified copy number alterations in recurrent pleomorphic adenoma (RPA) cases. The *x*-axis represents probes ordered according to their genomic position from chromosomes 1p to Xq. The *y*-axis denotes the log2 test/reference values (genomic gains in blue and losses in red are plotted above or below the 0 baseline, respectively; images adapted from the software Nexus Copy Number 7.0, Biodiscovery).

HMGA2 was observed in our cases. Overexpression of *HMGA2* from gene amplification seems to be present in malignant transformation of PA.[17] The only alteration found at chromosome 12 was gain at 12p13.2 encompassing the *ETV6* gene from 1 case of PA. Rearrangements of *ETV6* as *ETV6-NTRK3* or *ETV6-X* fusions were found in secretory carcinoma of the salivary glands, secretory carcinoma of the breast, and other tumors[18]; however, gains in *ETV6* have not been described in salivary gland tumors until now.

The most interesting finding of the present study was the somatic high copy number of *MAML2* and *LIFR*; amplification of these genes has not been described in salivary gland tumors until now. However, more cases presenting amplifications are necessary to draw further conclusions. *MAML2* is fused with *CRTC1* and *CRTC3* in mucoepidermoid carcinoma originating from t(11;19)(q21;p13) and t(11;15)(q21;q26), respectively.[19] The final product (fusion protein) influences the expression of other genes involved in the transformation of neoplastic cells.[20,21] In PAs, it was found that *LIFR* is a fusion partner of *PLAG1* resulting from t(5;8)(p13;q12), and this fusion leads to upregulation of *PLAG1* gene expression under the control of *LIFR*.[7] Therefore, *MAML2* and *LIFR* amplifications may be considered occasional findings, or they can be included in PA groups without known rearrangements.

In our series, no recurrence and malignant transformation were observed in the PA and RPA cases, and all patients are alive without disease. Carcinoma from RPA is reported in 1.5% to 23% of the cases, and when it occurs, it involves the acquisition of mutations over a period.[22] The risk of PA recurrence ranges from 4% to 45%, depending on the surgical technique.[23,24] Potential causative factors for recurrence are capsule rupture or incomplete resection of microscopic extensions beyond the pseudocapsule. Patient age ranges from 26 to 69 years (mean age 59.6 years), with no statistical difference in occurrence between males and females.[10] The time from the primary tumor to its recurrence ranged from 48 to 144 months, with a mean interval of 64.8 months.[10]

The recurrence of a tumor can be caused either by an increase in the number and complexity of gene abnormalities or by the acquisition of new mutations.[13] Clinical factors, surgical approach, and some markers are involved in RPA as upregulation of the expression of cell proliferation regulatory proteins.[12,13] Recently, de Brito et al.[13] found PLAG1 positivity in RPA by performing immunohistochemical analysis and concluded that expression of the protein is maintained from PA to RPA, suggesting that it may play a role in disease recurrence.

In this study, only 1 RPA case (25%) displayed changes in the number of copies (loss in 5p15.33-p15.1), whereas more than half the PAs (61.5%) presented some type of alteration, such as gene gain or amplification. According to our interpretation, this result may be a reflection of the small number of RPA samples studied (4 cases). This result, however, suggests that the change in the number of copies may not be responsible for recurrence and that other complex molecular mechanisms, such as translocations, gene fusions, or micro-RNA–mediated translation control, may play a more important role. Further studies are necessary for better understanding of the pathogenesis of these tumors.

CONCLUSIONS

Our data show that PA and RPA exhibit few somatic copy number alterations and that they show similar genomic profiles on aCGH, suggesting similar mechanisms of tumorigenesis. The copy number alteration at chromosome 8 and the amplified genes *MAML2* and *LIFR* may be a contributory factor in PA tumorigenesis. However, these genetic alterations might not play a role in tumor recurrence.

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