

PLAG1 gene alterations in salivary gland pleomorphic adenoma and carcinoma ex-pleomorphic adenoma: a combined study using chromosome banding, *in situ* hybridization and immunocytochemistry

Carmo Martins¹, Isabel Fonseca², Lúcia Roque¹, Teresa Pereira², Catarina Ribeiro¹, Jörn Bullerdiek³ and Jorge Soares²

¹*Centro de Investigação de Patobiologia Molecular (CIPM), Instituto Português de Oncologia de Francisco Gentil, Lisboa, Portugal;* ²*Serviço de Patologia Morfológica, Instituto Português de Oncologia de Francisco Gentil, Lisboa, Portugal and* ³*Center for Human Genetics, University of Bremen, Bremen, Germany*

Pleomorphic adenoma is the most common benign tumor of the salivary glands. It has marked histological diversity with epithelial, myoepithelial and mesenchymal-type cells arranged in a variety of architectural and differentiation patterns. *Pleomorphic adenoma gene 1 (PLAG1)*, shown to be consistently rearranged in pleomorphic adenomas, is activated by chromosomal translocations involving 8q12, the chromosome region that is most frequently affected in these tumors. In this study, we evaluated *PLAG1* involvement in salivary gland tumorigenesis by determining the frequency of its alterations in a selected group of 20 salivary gland tumors: 16 pleomorphic adenomas and four carcinomas ex-pleomorphic adenoma, having in common the presence of karyotypic chromosome 8 deviations, either structural, with 8q12 rearrangements, or numerical, with gain of chromosome 8. *PLAG1* status was analyzed using *in situ* hybridization techniques, on metaphase cells, by fluorescence detection and/or interphase cells in paraffin sections, by chromogenic detection. Except for one pleomorphic adenoma case (5%) that lacked *PLAG1* involvement, 17 tumors (85%), (14 pleomorphic adenomas and three carcinomas ex-pleomorphic adenoma) showed intragenic rearrangements of *PLAG1* and the remaining two cases (10%), (one pleomorphic adenoma and one carcinoma ex-pleomorphic adenoma), had chromosome trisomy 8 only. To further investigate the role of *PLAG1* on pleomorphic adenomas tumorigenesis, as well as the putative morphogenesis mechanism, we attempted to identify the cell types (epithelial vs myoepithelial) carrying 8q12/*PLAG1* abnormalities by a combined phenotypic/genotypic analysis in four cases (three pleomorphic adenoma and one carcinoma ex-pleomorphic adenoma) characterized by 8q12 translocations and *PLAG1* rearrangement. In these cases, both cells populations carried *PLAG1* rearrangements. This finding further supports the pluripotent single-cell theory, which postulates that the tumor-initiated, modified myoepithelial cell, evolves into the varied somatic cell phenotypes present in pleomorphic adenoma, and reinforces the role of *PLAG1* on the tumorigenesis of benign and malignant pleomorphic adenoma.

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Pleomorphic adenoma, the most common salivary gland tumor type, shows a marked histological diversity which reflects the epithelial and mesenchymal cell differentiation in its composition.^{1,2}

Extensive cytogenetic studies have revealed that these tumors have highly specific chromosome abnormalities namely 3p21, 8q12 and 12q13–15 rearrangements and trisomy 8.^{3–6}

Among them, 8q12 deviations constitute the largest cytogenetic subgroup, the most frequent structural aberration being the reciprocal translocation t(3;8)(p21;q12). Molecular characterization of the t(3;8) translocation allowed the identification of two genes, *PLAG1* and *CTNNB1* involved at 8q12 and 3p21, respectively.⁷ The former is a

Correspondence: Dr C Martins, PhD, CIPM-Cytogenetic Laboratory, Portuguese Cancer Institute, R. Prof Lima Basto, Lisboa, Portugal.

E-mail: mcmartins@ipolisboa.min-saude.pt

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developmentally regulated zinc-finger gene, activated by a promoter swapping mechanism, in which the *PLAG1* promoter is replaced by the *CTNNB1* gene promoter.⁷ Recently, it was demonstrated that *PLAG1* gene acts as a genuine proto-oncogene⁸ and that its overexpression, with deregulation of *PLAG1* target genes, is a crucial oncogenic event in pleomorphic adenomas.⁹ Rearrangements of *PLAG1* and tissue overexpression were also reported in a large percentage of pleomorphic adenomas showing other molecular 8q12 rearrangement and even in cases without cytogenetically detectable 8q12 aberrations.^{7,10–12}

The role of *PLAG1* in salivary gland tumors is almost confined to pleomorphic adenomas as well as to carcinomas ex-pleomorphic adenomas arising in them.^{10,13,14}

We investigated the status of *PLAG1* gene on a selected group of 20 salivary gland tumors comprising 16 pleomorphic adenomas and four carcinomas ex-pleomorphic adenoma, which share karyotypic chromosome 8 deviations, either as structural (8q12 rearrangements) or numerical gains. This study was performed using different *in situ* hybridization techniques: in metaphase cells, the fluorescence detection (FISH), and/or in interphase cells from formalin-fixed, paraffin-embedded tumor sections, the chromogenic detection (CISH). CISH analysis was aimed at verifying intragenic rearrangements of *PLAG1* in uncultured tumor cells, *in situ*, and at comparing the results obtained with both *in situ* hybridization approaches.

The histogenesis of pleomorphic adenoma remains a controversial issue, two main theories emerging, the one that explains pleomorphic adenoma as the result of clonal expansion of a single pluripotent cell,^{15,16} and the other which considers that pleomorphic adenoma and other types of biphasic salivary tumors arise from the coordinated growth of cell populations derived from cells with maintained proliferating capability in salivary tissue.¹⁷ Clonality has been previously demonstrated in pleomorphic adenoma,^{18–20} the studies supporting the view favoring the common pluripotent cell theory for the salivary gland neoplasm. Debiec-Rychter *et al*¹⁸ using *PLAG1* genetics and immunohistochemistry demonstrated that the gene lesions were present in all tumor cell populations of pleomorphic adenoma, though more distinctly expressed in cells showing mesenchymal phenotype. They postulated that neoplastic cells with a clearly demonstrable epithelial phenotype probably evolve from cells with mesenchymal characteristics.¹⁸

Aiming at clarifying this issue, and to further elucidating the role of *PLAG1* in pleomorphic adenoma morphogenesis, we searched, by a combined genotypic and phenotypic analysis, whether the (cyto)genetic aberrations were expressed by the epithelial cells, the myoepithelial cells or both, using a combination of immunocytochemistry and *in situ* hybridization techniques, applied to the

study of three cases of pleomorphic adenomas and one carcinoma ex-pleomorphic adenoma, all of them presenting a 8q12 translocation.

Materials and methods

Case Selection

In all, 20 previously karyotyped cases of salivary gland pleomorphic adenoma were selected: 16 benign tumors and four with associated carcinoma areas. All of them had in common chromosome 8 deviations, either as structural rearrangements affecting 8q12 region (90% of the cases), or as numerical gain of chromosome 8 (10% of the cases). One of the benign neoplasms (case 1) had morphologic features fitting with the diagnosis of myoepithelioma. The clinical, histopathological and karyotypic information are provided in Table 1.

Conventional Cytogenetic Analysis

Chromosome metaphases of tumor cells were obtained from short-term primary cultures as previously described.²¹ Chromosomes were GTG-banded, and the karyotypes were established according to the ISCN rules.²² Detailed description of the tumor karyotypes can be retrieved from Table 1.

In Situ Hybridization—FISH and CISH Analysis

FISH analysis

FISH analysis was performed on metaphase chromosomes using a set of three PAC clones covering the region of *PLAG1* gene. PAC clones DNAs were isolated as described by Rogalla *et al*²³ and labelled, simultaneously, with biotin by random octamer priming, using the Bioprime DNA labelling system (Life Technologies Inc.). A standard FISH protocol was applied.²⁴ Biotinylated *PLAG1* probe was detected by Cy3-avidin (Jacksons Lab) and chromosomes were counterstained with DAPI-Vectashield mounting solution (Vector). Fluorescence hybridization signals were analyzed and recorded using a Cytovision System (Applied Imaging).

CISH analysis

CISH was performed on 4 µm formalin-fixed paraffin-embedded tissue sections, containing both the normal salivary gland tissue and the neoplastic tissue. Two probes were used: the *PLAG1* probe and a (peri)centromeric probe for chromosome 8 (cen8) (ATCC). The centromeric 8 probe was used both to confirm trisomy of chromosome 8 and as a control probe.

CISH analysis was performed as previously described by Alers *et al*.²⁵ CISH probes were labelled with fluorescein (Amersham Pharmacia), by random priming, as described above. Probe detection was

Table 1 Clinical, histopathological and karyotypic data from 20 salivary gland tumors with chromosome 8 deviations

Case	Classification	Age/sex	Location	Karyotype
1	Pleomorphic adenoma	57/F	Parotid	45–46,XX,t(2;8)(p23;qq22),t(8;12)(q12;p13)[cp8]/46,XX[3]
2	Pleomorphic adenoma	45/M	Parotid	46,XY,t(8;9)(q12;p22–23)[7]
3	Pleomorphic adenoma	68/F	Palate	46,XX,t(5;8)(p13;q12–13)[8]
4	Pleomorphic adenoma	51/M	Parotid	46,XY,t(8;10)(q12;q22)[13]
5	Pleomorphic adenoma	78/M	Parotid	46,XY,t(3;8)(p21;q12)[11]/45,XY,t(3;8)(p21;q12),der(13;15)(q10;q10)[2]/46,XY[2]
6	Pleomorphic adenoma	37/F	Submandibular	46,XX,t(8;15)(q12;q26)[13]
7	Pleomorphic adenoma	22/F	Parotid	46,X,t(X;2)(q26;q21),t(3;8;9)(p21;q12;p21)[9]
8	Pleomorphic adenoma	86/F	Submandibular	46,XX,t(3;8)(p21;q12)[16]
9	Pleomorphic adenoma	72/F	Palate	46,XX,t(3;8)(p21;q12)[6]
10	Pleomorphic adenoma	24/F	Parotid	46,XX,t(3;8)(p21;q12)[7]/46,XX[11]
11	Pleomorphic adenoma	73/F	Parotid	46,XX,t(3;8)(p21;q12)[8]
12	Pleomorphic adenoma	40/F	Parotid	46,XX,t(8;9)(q12;p22–23)[8]
13	Pleomorphic adenoma	34/M	Parotid	45 ~ 46,XY,t(3;8)(p21;q12)[cp11]
14	Pleomorphic adenoma	68/F	Parotid	44 ~ 46,XX,t(3;8)(p21;q12)[cp6]
15	Pleomorphic adenoma	56/F	Parotid	47,XX,+8[11]/46,XX[15]
16	Pleomorphic adenoma	33/F	Parotid	46,XX,t(5;8)(p15;q12)[6]/46,XX[10]
17	Carcinoma ex-pleomorphic adenoma	48/M	Parotid	46,XY,t(3;8)(p21;q12)[10]
18 ^a	Carcinoma ex-pleomorphic adenoma	71/F	Submandibular	105–111,XXXXX,–1,i(1)(q10),del(2)(q13),+3,der(3)t(3;8)(p21;q12) × 4,+6,+7,+7,+7,der(8)i(8)(q10)t(3;8)(p23;q12)x2,+der(?)t(1;9)(p22;?) × 2,–10,–11,–11,der(11)t(11;11)(p15;q13)x3,del(12)(p12),–13,–14,–15,–16,del(16)(q22) × 2,17,–18,–18,–18,i(18)(q10),+19,–21,+22[cp8]/46,XX[3]
19	Carcinoma ex-pleomorphic adenoma	58/M	Parotid	47,XY,+8[10]/45,X,–Y[6]/46,XY[6]
20	Carcinoma ex-pleomorphic adenoma	60/F	Parotid	46,XX,ins(3;8)(p21;q12qter)[10]

^aCase previously published (Jin *et al*¹³).

performed using horseradish peroxidase antiluorescein (Roche) and H₂O₂-diaminobenzidine. Tissue sections were counterstained with hematoxylin and mounted with Entellan (Merck).

In each case, per tissue section, both probes (cen8 and *PLAG1*) were evaluated in tumor and normal tissue areas. For *PLAG1* probe, the number of cells that were analyzed varied between 69 and 237 (mean = 197) in tumor areas (15 cases) and between 65 and 218 (mean = 174) in normal areas (12 cases). Cen8 probe was evaluated in tumor areas (16 cases), the range being 183–218, (mean = 209) and in normal areas (16 cases) from 115 to 221 (mean = 195). The number of spots per nucleus was scored as 1, 2, 3, 4 or >4 in all cases, excepting one case of carcinoma ex-pleomorphic adenoma, which was aneuploid and in which the range used was 1 to ≥10.

In normal areas, in the assessment of cen8 probe the mean percentage of nuclei (mean ± s.d.), containing 1, 2 and 3 hybridization signals per nucleus, was, respectively, 30.5 ± 15.6, 68.7 ± 15.3 and 0.7 ± 1.1. Similarly the following values: 28.0 ± 32.8, 70.4 ± 28.7 and 1.7 ± 1.1 signals, respectively, were found for *PLAG1* probe.

Significant abnormal losses (monosomy) or gains (polisomy) evaluated with the centromeric probe used were considered to be present when the percentage of samples with one or ≥3 signals per nucleus was higher than the mean ± 3 s.d. of the controls.²⁶ We adopted the conservative criteria proposed by Jenkins *et al*²⁷ in paraffin-embedded

tissue sections, assuming that ≥10% of the nuclei with three signals for cen8 and *PLAG1* probes, respectively, as trisomy 8 or split of the *PLAG1* gene.

Combined Immunocytochemistry and *In Situ* Hybridization Analysis

To investigate which tumor differentiated cell type carried the specific cytogenetic lesions, a combined approach of phenotypic and genotypic analysis was performed on paraffin tissue tumor sections, using, respectively, immunocytochemical and FISH techniques. The phenotype of tumor cell populations was established using mouse monoclonal antibodies against calponin (dilution 1:600) (Dako) and cyto-keratin CK8-18 (dilution 1:10) (Novocastra) as markers of myoepithelial and epithelial cells, respectively. The slides were incubated for 30 min at room temperature with the primary antibodies, after antigen retrieval using pressure cooking for 1 min in citrate buffer (pH = 6.0). The reaction was developed with Cy3-avidin (Jacksons Lab).

Subsequently, FISH was performed, on the same slides, using the *PLAG1* probe and the centromeric 8 probe as an internal control. The *PLAG1* probe was labelled with digoxigenin and centromeric 8 probe with biotin and detected, respectively, by antidigoxigenin-FITC (Roche) and Cy3-avidin. The nuclei were counterstained with DAPI. Immunocytochemistry and FISH results were analyzed at the same time and recorded using the Cytovision System.

Results

Evaluation of *PLAG1* Gene Alterations by *In Situ* Hybridization (FISH and CISH Analysis)

PLAG1 gene evaluation was feasible in all tumors by metaphase FISH (18 cases) and/or paraffin-section interphase CISH (16 cases) using the *PLAG1* probe and the (peri)centromeric probe for chromosome 8 (Table 2).

Intragenic rearrangement of *PLAG1* was observed in nine pleomorphic adenoma and three carcinoma ex-pleomorphic adenoma cases by FISH analysis, recognized by hybridization signals of *PLAG1* probe on rearranged chromosomes 8 and their translocation partners (split signal). No split signal was detected in cases 10, 11 and 12. Cases 10 and 11, which carried a typical t(3;8)(p21;q12) did not show the expected rearrangement of *PLAG1* gene. Only two signals were observed: one at der(8) and one at the normal chromosome 8. There was no signal at the translocation partner der(3). The signal on der(8) appeared smaller than the signal on normal chromosome 8, indicating that a deletion of 3p sequences along with the t(3;8) had presumably occurred. In the two cases showing trisomy 8 (cases 15 and 19), three copies of *PLAG1* were detected without *PLAG1* region rearrangement. In cases 1 and 9, it was not possible to assess *PLAG1* status due to lack of metaphases.

By CISH analysis, *PLAG1* could be evaluated in 15 cases, confirming split of *PLAG1* in a significant number of nuclei, which displayed three signals on FISH-positive cases and evidenced *PLAG1* rearrangements on unprobed *PLAG1* cases 1 (20.1%) and 9 (29.5%) (Table 2). Except for case 19, that showed 21.6% of nuclei with three signals/copies confirm-

ing trisomy 8, all the other cases probed with the centromeric 8 probe were disomic.

In cases 10 and 11, which were 'split *PLAG1* negative by FISH', the three signal counts per nucleus were, respectively, 9.7 and 14.0% of the nuclei analyzed. These values fulfilled the adopted criteria of $\geq 10\%$ for *PLAG1* split, comprehensively on case 11 and at the lower limit for case 10. These two cases were interpreted as positive for *PLAG1* rearrangement. CISH confirmed the absence of intragenic rearrangement of *PLAG1* in case 12, which was characterized by a t(8;9)(q12;p22-23) with breakpoint at 8q12. FISH analysis of this case with painting probes (data not shown) confirmed the translocation, being excluded the involvement of other(s) chromosome(s).

Case 3 displayed a more complex rearrangement of *PLAG1*. G-band analysis revealed what appeared to be a balanced translocation t(5;8)(p13;q12-13), that was confirmed by FISH using painting probes for chromosome 5 and 8 (Figure 1a and b). However, when *PLAG1* status was investigated, instead of finding the expected *PLAG1* split with signals on both der(5) and der(8), two small signals from *PLAG1* on der(8), and absence of signal on der(5) were verified, which indicates that a complex cytogenetic rearrangement had probably occurred (Figure 1c). The same pattern was clearly detected by CISH in paraffin sections (Figure 1d).

Identification of Cell Types Carrying 8q12 Rearrangements by a Combined Genotypic/Phenotypic Analysis

Immunocytochemical techniques to assess calponin and cytokeratin immunoreactivity were applied to

Table 2 Cytogenetic and *in situ* hybridization data from 20 salivary gland tumors with chromosome 8 deviations

Case	Cytogenetic	FISH <i>PLAG1</i>	CISH/No of signals from <i>PLAG1</i>	CISH/No of signals from <i>cen8</i>
1	t(8;12)	Not done	1(26.9%):2(50.5%):3(20.2%):4(2.4%) split	1(32.4%):2(66.2%):3(0.9%):4(0.5%)
2	t(8;9)	Split	1(11.2%):2(48.0%):3(38.3%):4(1.5%):>4(1.0%) split	1(28.2%):2(70.5%):3(1.3%)
3	t(5;8)	Split	1(19.4%):2(24.8%):3(55.3%):4(0.5%) split	1(33.8%):2(65.3%):3(0.9%)
4	t(8;10)	Split	1(24.0%):2(44.7%):3(30.3%):4(1.0%) split	1(32.4%):2(66.6%):3(1.0%)
5	t(3;8)	Split	No results	No results
6	t(8;15)	Split	1(24.9%):2(44.6%):3(28.7%):4(1.3%):>4(0.5%) split	1(33.6%):2(64.5%):3(1.4%):4(0.5%)
7	t(3;8;9)	Split	1(19.5%):2(38.5%):3(38.5%):4(3.5%) split	1(30.0%):2(69.0%):3(1.0%)
8	t(3;8)	Split	1(17.4%):2(63.7%):3(17.4%):4(1.5%) split	1(30.0%):2(68.9%):3(1.1%)
9	t(3;8)	Not done	1(26.5%):2(43.0%):3(29.5%):4(1.0%) split	1(32.0%):2(66.5%):3(1.5%)
10	t(3;8)	No split	1(19.0%):2(70.8%):3(9.7%):4(0.5%) split	1(32.2%):2(67.3%):3(0.5%)
11	t(3;8)	No split	1(21.5%):2(61.5%):3(14.0%):4(3.0%) split	1(38.0%):2(62.0%)
12	t(8;9)	No split	1(25.3%):2(69.7%):3(3.5%):4(1.5%) no split	1(37.5%):2(62.5%)
13	t(3;8)	Split	1(9.6%):2(46.9%):3(41.5%):4(2.0%) split	1(22.5%):2(75.6%):3(1.9%)
14	t(3;8)	Split	1(18.0%):2(45.5%):3(33.8%):4(2.7%) split	1(30.5%):2(67.5%):3(2.0%)
15	+8	Trisomic	No results	No results
16	t(5;8)	Split	No results	No results
17	t(3;8)	Split	1(11.4%):2(50.0%):3(34.1%):4(4.5%) split	1(32.3%):2(63.7%):3(3.0%):4(1.0%)
18	der(8q12)	Split	1(2.3%):2(13.3%):3(6.8%):4(9.6%):5(13.8%):6(14.7%):7(12.0%):8(10.5%):9(9.2%):10(3.2%):>10(4.6%) split	1(7.2%):2(19.0%):3(18.4%):4(16.2%):5(19.8%):6(16.2%):7(3.15%)
19	+8	Trisomic	No results	1(18.8%):2(47.6%):3(21.6%):4(12.0%)
20	ins(3;8)	Split	No results	Trisomic No results

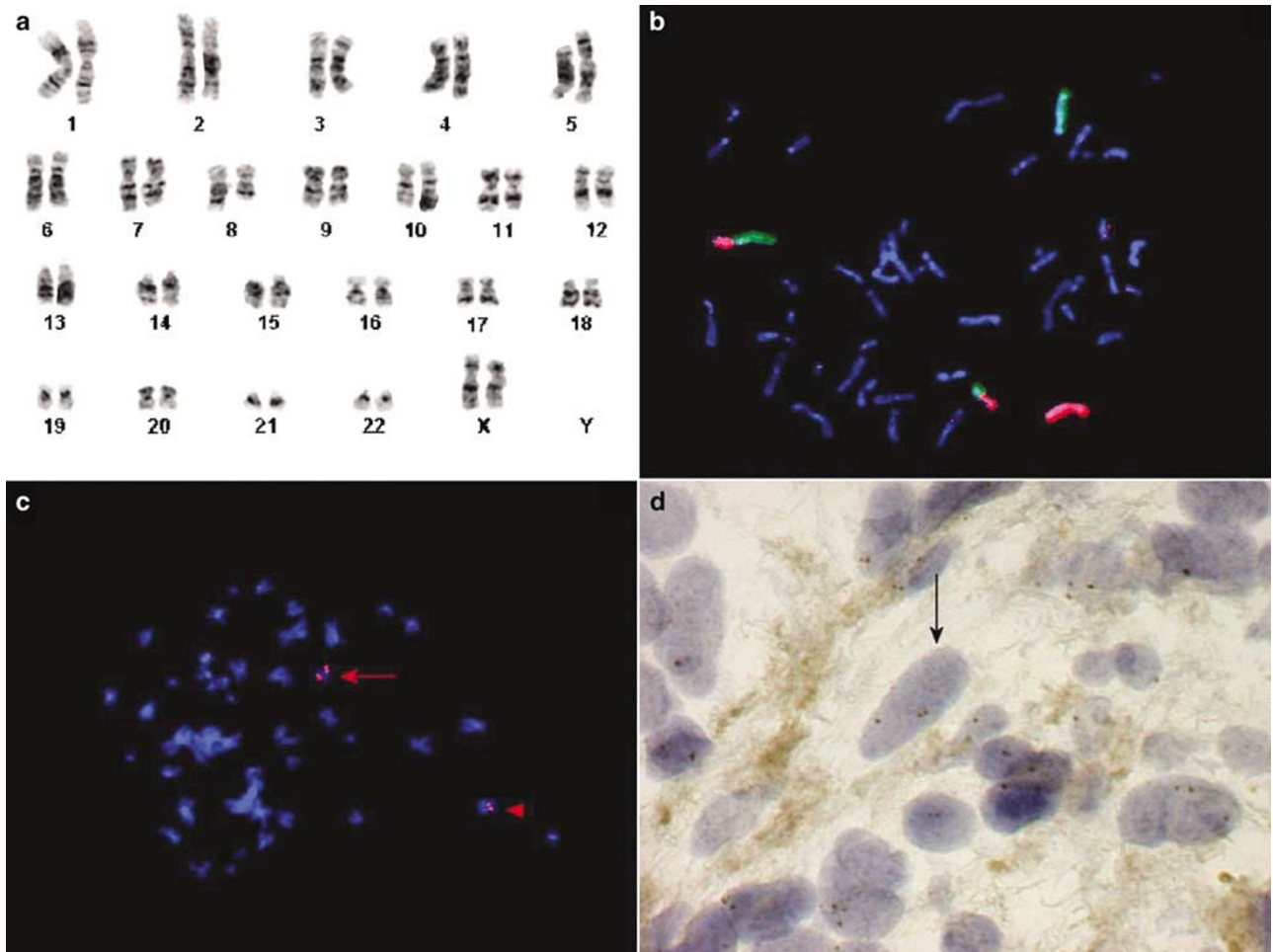


Figure 1 G-band and *in situ* hybridization analysis of a pleomorphic adenoma (case 3) with a t(5;8)(p13;q12-13). (a) Representative karyotype of the case; (b) FISH analysis with painting probes for chromosome 5 (green) and chromosome 8 (red), confirming the translocation; (c) FISH analysis with *PLAG1* probe (red) with signal on normal chromosome 8 (arrow head) and with two small signals on the der(8) (arrow); (d) CISH analysis on a paraffin section, with *PLAG1* probe; there are two adjacent brown spots, and a third spot confirming FISH observations.

investigate myoepithelial and epithelial differentiation of tumor cells from pleomorphic adenoma cases 2, 3, 4 and carcinoma ex-pleomorphic adenoma case 17, all of them characterized by 8q12 translocations and *PLAG1* rearrangement. In order to establish the genotype of the cell population, the same slides were processed for FISH analysis using the *PLAG1* probe. In all cases, the *PLAG1* gene was found rearranged in either calponin- or cytokeratin-positive cells demonstrating that the (cyto)genetic lesion was shared by both myoepithelial and epithelial cells. Figure 2(a,b) illustrates in case 4 *PLAG1* rearrangement in both cytokeratin- and calponin-positive cells.

Discussion

Our study describes a sizeable series of salivary gland pleomorphic adenomas, having in common 8q12 deviations and *PLAG1* involvement, assessed by *in situ* hybridization techniques. Both metaphase and interphase tumor cells were evaluated which

represents a new methodological approach using archival, formalin-fixed paraffin-embedded tissue. Additionally, it provides information on carcinoma ex-pleomorphic adenoma, a rare neoplasm in which very scarce information about the *PLAG1* status is available.

This homogeneous series includes 17 tumors (85%), being 14 pleomorphic adenomas and three carcinomas ex-pleomorphic adenoma, all showing intragenic rearrangements of *PLAG1*. Two tumors (10%) had chromosome 8 trisomy without molecular cytogenetic evidence of *PLAG1* rearrangement. Only pleomorphic adenoma case 12, which is characterized by a t(8;9)(q12;p22-23), with breakpoint at 8q12 lacked *PLAG1* involvement. FISH analysis of this case performed with painting probes confirmed the translocation and excluded the involvement of other chromosome(s). *In situ* hybridization analysis performed either in metaphase or interphase cells revealed only two signals/copies of *PLAG1* which indicates that no disruption of *PLAG1* gene had occurred. In a recent study, Røijer *et al*²⁸

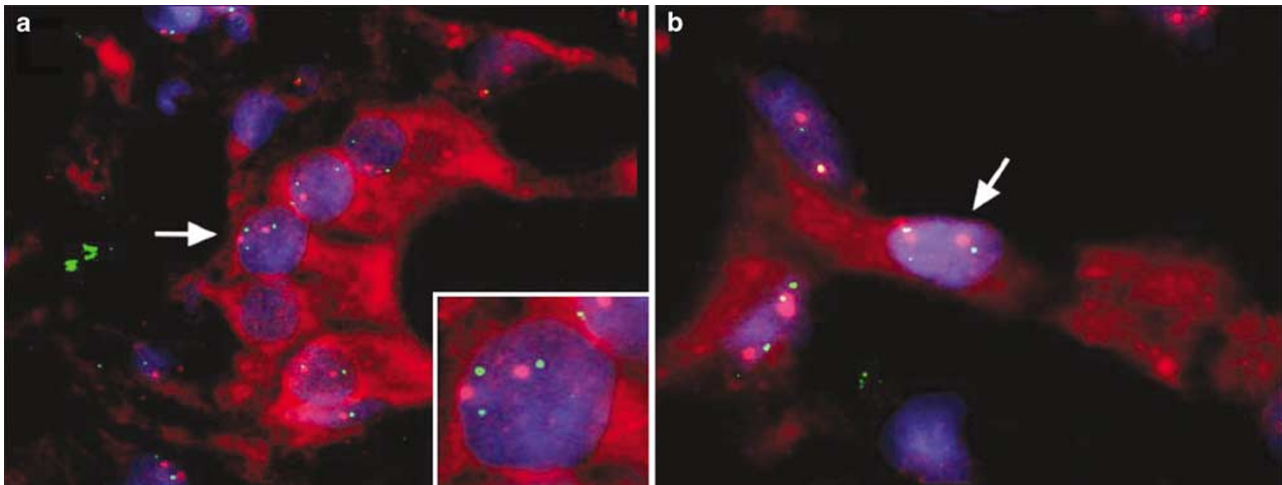


Figure 2 Combined immunocytochemistry and FISH analysis of a pleomorphic adenoma (case 4) with rearrangement of *PLAG1* gene. (a) Cytokeratin (CK8-18)-positive cell (white arrow) showing the rearranged *PLAG1* gene: three green spots can be observed corresponding one to the normal *PLAG1* gene and two to the rearranged *PLAG1* gene. Two red spots corresponding to two centromeres of chromosome 8 are present. (b) Calponin-positive cell (white arrow) also showing the rearrangement of *PLAG1* gene (green).

described three pleomorphic adenoma cases with 8q12-13 rearrangements also without *PLAG1* alteration. Further characterization of these cases will potentially shed light into the mechanisms behind these rearrangements.

PLAG1 involvement has been mainly reported in pleomorphic adenoma cases associated with 8q12 deviations, but it has also been shown to be activated in cases of pleomorphic adenoma either with normal karyotypes or with 12q13-15 abnormalities.^{7,10-12} In contrast to benign neoplasm, rearrangement of *PLAG1* was only observed in a limited number of malignant tumors, mainly cases of carcinoma ex-pleomorphic adenoma.^{10,13,14}

In the present study, except for one case (# 12), all pleomorphic adenomas with 8q12 deviations had an intragenic rearrangement of *PLAG1*, which confirms the high percentage of this genetic event in pleomorphic adenoma and confirms its central role in salivary gland tumorigenesis.

Carcinomas ex-pleomorphic adenoma are uncommon neoplasm representing about 12% of all malignant salivary gland tumors.²⁹ By definition they occur in the context of a previous or synchronous pleomorphic adenoma and they usually pursue an aggressive course.²⁹ Histologically, they are characterized by the presence of benign and malignant areas, the latter usually having features of high-grade adenocarcinoma.² Previous cytogenetic and molecular studies revealed that carcinomas ex-pleomorphic adenoma have recurrent rearrangements at 8q12 and 12q13-15, the same chromosome regions affected in pleomorphic adenoma, and they also show consistent alterations at 17p loci (see El-Naggar *et al*³⁰ for review). Consequently, *PLAG1* gene rearrangement is expected to occur in cases of carcinoma ex-pleomorphic adenoma with 8q12 deviations. In this study, we found that the three

8q12 rearranged carcinoma ex-pleomorphic adenoma cases showed *PLAG1* rearrangement which corroborates the involvement of this gene in tumor development.

The trisomy 8 is known to be the most frequent numerical deviation found in salivary gland tumors, being present in benign and malignant tumors.³¹ In the present series, gain of chromosome 8 was the sole karyotypic aberration found in one case of pleomorphic adenoma and in one case of carcinoma ex-pleomorphic adenoma. The FISH analysis performed demonstrated that *PLAG1* region was not structurally affected, since the two tumors had only one copy number gain of the gene. The mechanisms associated with gain of chromosomes are still unclear; the most plausible explanation is that gene dosage alterations predispose to tumor proliferation. A novel oncogenic mechanism involving *PLAG1* copy number gain was described in the study of a series of lipoblastomas that presented polysomy for chromosome 8 without *PLAG1* rearrangement.³² In this series, it was demonstrated that the 8q12 chromosomal rearrangements result in upregulated expression of *PLAG1* transcription factor. It was hypothesized that, in polysomy 8/nonrearranged *PLAG1* cases, *PLAG1* could be transcriptionally upregulated, through a mechanism similar to the one verified in lymphoid neoplasms, such as Burkitt's lymphoma, in which point mutations in the promoter-region of *MYC* oncogene were detected in fusion gene-negative cases (see Gisselsson *et al*³² for review). Whether or not an analogous mechanism is present in salivary gland benign and malignant mixed tumors it remains to be elucidated.

One of the aims of our study was to compare the utility of *in situ* hybridization techniques applied to metaphase and interphase tumor cells using archived paraffin-embedded tissue and a specific-gene

probe for the detection of gene rearrangements. Karyotyping mandates the establishment of a cell culture *in vitro* using a fresh sample of the primary tumor. In contrast, the *in situ* hybridization technique performed on paraffin sections, makes it possible to evaluate the *in situ* genetic alterations, in uncultured cells, avoiding *in vitro* manipulation and the need for fresh tissue. We found a fairly good agreement between the two techniques which allow to recommend their use in future studies. The *in situ* hybridization analysis of paraffin sections was shown to be a very sensitive technique as demonstrated in case 3. Karyotypically, this case suggested the existence of a simple translocation between chromosomes 5 and 8 in the form of t(5;8)(p13;q12), an observation also confirmed by FISH with painting probes for both chromosomes. However, when *PLAG1* status was evaluated by FISH in metaphase cells, two small spots from *PLAG1* on der(8) were observed but none on der(5), prompting to consider that a more complex rearrangement occurred, such an inversion on chromosome 8, which initially splits the *PLAG1* locus, along with a translocation with chromosome 5. A similar phenomenon was also observed in pulmonary hamartomas of the lung, where hidden chromosomal inversions occurring together with simple translocations were also disclosed by FISH analysis.³³ CISH analysis on paraffin sections of this case revealed the same pattern, demonstrating that the *in situ* hybridization analysis is a powerful tool to assess *PLAG1* rearrangements on paraffin-embedded archival tissues.

The second question addressed by this study was the investigation of the role of *PLAG1* in the tumorigenesis and differentiation of pleomorphic adenomas. These neoplasms are morphologically characterized by a biphasic pattern of differentiation illustrated by the coexistence of epithelial and mesenchymal areas. Controversy remains on whether pleomorphic adenoma arises from a single pluripotent cell or more than one 'stem cell' is involved in the mechanism of clonal tumor expansion.¹⁷ There is increasing evidence that epithelial and myoepithelial cell populations share phenotypical and genotypical characteristics supporting the 'modified myoepithelial cell model' for pleomorphic adenoma histogenesis.^{16,17}

Identification of cell types carrying *PLAG1* rearrangements could help to clarify this issue. This prompted us to perform a combined phenotypic/genotypic analysis on pleomorphic adenoma and carcinoma ex-pleomorphic adenoma cases of this series characterized by 8q12 translocations and *PLAG1* intragenic rearrangement. In all cases, we identified cells expressing myoepithelial and ductal epithelial features. The genotype of the cells was established using FISH techniques, for the localization of 8q12 abnormalities that was verified in both calponin- and keratin-positive cells, which points to accept that the cytogenetic lesion is shared by epithelial and myoepithelial cell lineages. Further

evidence to this conclusion came from the study of Debiec-Richter *et al*¹⁸ in two cases of pleomorphic adenoma characterized by 8q12 translocations. In this study, they investigated, which cell types expressed *PLAG1* protein and they demonstrated that all cells that were strongly immunoreactive for *PLAG1* carried the specific chromosomal translocations, and that they phenotypically, were either epithelial or myoepithelial. Our results and these data reinforce, therefore, the theory that postulates that pleomorphic adenoma cells originate in a single cell, a putative pluripotent element capable of differentiation into a variety of somatic phenotypes, as the model proposed by Batsakis and El-Naggar.¹⁶

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