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PLAG1 immunohistochemistry is a sensitive marker for Pleomorphic Adenoma: a comparative study with *PLAG1* genetic abnormalities

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Abstract

Aims—*PLAG1* gene rearrangement is the most common genetic abnormality in pleomorphic adenoma (PA), resulting in overexpression of PLAG1 protein. PA and carcinoma ex pleomorphic adenoma (CA ex-PA) can mimic various benign and malignant salivary gland tumors. The aims of this study are to evaluate the sensitivity and specificity of PLAG1 immunohistochemistry (IHC) in the differential diagnosis of PA and CA ex-PA and to compare the PLAG1 immunohistochemical results to *PLAG1* gene abnormalities as detected by fluorescence in situ hybridization (FISH).

Methods and Results—PLAG1 immunostaining was performed on 83 salivary gland tumors including 23 PA, 15 CA ex-PA, and 45 other salivary gland tumors. In addition, *PLAG1* FISH was performed in 44 cases for the presence of gene rearrangements/amplifications. The results showed high sensitivity of PLAG1 IHC in 96% of PA; however, discordant results between *PLAG1* FISH abnormalities and IHC were noted in 15/44 cases (34%). Seven PA, four de novo myoepithelial carcinomas and one basal cell adenocarcinoma had negative FISH results, but were positive for IHC; while 3 salivary duct carcinomas (SDC) ex-PA were positive for FISH but negative for IHC. PLAG1 IHC can differentiate CA ex-PA from de novo SDC (p=0.02), but not from de novo myoepithelial carcinoma. PLAG1 IHC is a sensitive marker for PA. This could be due to *PLAG1* gene abnormalities beyond FISH resolution.

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Authors' contributions:

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Conclusions—A negative PLAG1 IHC might be helpful in excluding a PA diagnosis. Interestingly in the context of CA ex-PA, FISH is more sensitive than IHC in detecting PLAG1 abnormalities.

Keywords

PLAG1; immunohistochemistry; fluorescence in situ hybridization (FISH); pleomorphic adenoma; salivary gland

INTRODUCTION

Pleomorphic adenoma (PA) is the most common salivary gland neoplasm, accounting for approximately 60% of all epithelial salivary gland tumors [1]. Histologically, PA is typically composed of three components of various proportions: epithelial/ductal cells, myoepithelial cells, and myxoid/chondroid stroma [2]. However, as PA is known to be associated with marked morphological diversity, distinguishing PA from other salivary gland neoplasms, benign or malignant, may not always be straightforward and may require additional ancillary studies (e.g. immunohistochemistry, cytogenetics, and molecular analysis).

Pleomorphic adenoma gene 1 (*PLAG1*) is a zinc finger transcription factor and a proto-oncogene located on chromosome 8q12. Fusions involving *PLAG1* locus were first discovered in PA in 1997 [3], and has since been reported in 24 to 88% of PA and carcinoma ex-pleomorphic adenoma (CA ex-PA) [2, 4–7]. *PLAG1* fusion appears to be highly specific for PA and CA ex-PA as it has not been detected in other benign or malignant salivary gland neoplasms [2, 4–8]. The high prevalence and near 100% specificity of *PLAG1* fusion make it an attractive potential target of ancillary diagnostic tests. As *PLAG1* primers for polymerase chain reaction (PCR) and *PLAG1* probes for fluorescence in situ hybridization (FISH) are not readily available commercially and as molecular and cytogenetics diagnostic laboratory services are usually provided only in large academic centers, there is a demand to seek for an affordable and feasible alternative testing method for *PLAG1* translocation.

Fusions between *PLAG1* gene and various partners, including *CTNNB1* (β-catenin), *LIFR* (leukemia inhibitory factor receptor), *FGFR1* (fibroblast growth factor receptor 1), and *CHCHD7* (Coiled-Coil-Helix-Coiled-Coil-Helix Domain Containing 7), leads to overexpression of PLAG1 oncoprotein [2, 4–7]. PLAG1 overexpression mediate multiple downstream factors, including the insulin-like growth factor 2 (IGF-2) mitogenic signaling pathway, growth factors and their receptors, tumor suppressors, cell cycle-related proteins, and apoptosis-related proteins, which subsequently influence cell proliferation and tumorigenesis [9–13]. Additionally, *PLAG1* alteration has been reported in skin and soft tissue myoepithelioma [14, 15], lipoblastoma [16], hepatoblastoma [17], uterine leiomyoma [18], uterine endometrial stromal sarcoma [19], and certain types of acute myeloid leukemia [10].

Several recent studies have shown that PLAG1 overexpression can be detected by immunohistochemistry (IHC), and that PLAG1 IHC is a relatively sensitive marker for PA in surgical and cytologic samples [4, 20–26]. However, most of these studies did not include molecular or cytogenetics assays as the gold standard reference test to confirm the presence

of *PLAG1* fusion and to evaluate the sensitivity and specificity of PLAG1 IHC in detecting *PLAG1* fusion.

In the present study, we aimed to characterize PLAG1 IHC in a large cohort of 83 salivary gland tumors, including PA, CA ex-PA, and other types of salivary gland tumors. Moreover, a significant subset of these tumors was examined for *PLAG1* fusion in order to determine the sensitivity and specificity of PLAG1 IHC in detecting *PLAG1* fusion and in differentiating PA and CA ex-PA from other potential mimickers.

MATERIAL AND METHODS

Case selection and study cohort

Eighty-three patients with epithelial salivary gland neoplasms who had surgery at Memorial Sloan Kettering Cancer Center (New York, NY) between 1995 and 2014 with appropriate material for subsequent IHC and FISH studies were included. The histologic slides were reviewed by a head and neck pathologist (NK) to confirm the diagnosis. The study was approved by the Institutional Review Board of MSKCC. Informed consent was not required for this retrospective study.

Tumor histology

The study cohort was composed of 23 PAs, 15 CAs ex-PA, and a control group of 45 other types of salivary gland carcinomas without a PA component (including ten de novo myoepithelial carcinomas (MECA), six de novo salivary duct carcinomas (SDC), four basal cell adenocarcinomas, eight polymorphous adenocarcinoma (PAC), two epithelial-myoepithelial carcinomas (EMC), four mucoepidermoid carcinomas (MEC), five adenoid cystic carcinomas (ACC), five acinic cell carcinomas (AciCC), and one secretory carcinoma (previously known as mammary analogue secretory carcinoma) (Table 2). The histologic subtypes of the CAs ex-PA were as follow: SDC (n = 9), MECA (n = 3), carcinoma with squamous and glandular features (n = 1), EMC (n = 1), and one adenocarcinoma not otherwise specified (NOS) (n =1).

PLAG1 immunohistochemistry

Immunohistochemical stains for PLAG1 were performed using monoclonal antibody clone 3B7 (4ug/ml; Novus Biologicals, Littleton, CO). All immunostains were done on a Leica Bond-3 (Leica, Buffalo Grove, IL) automated stainer platform. Prior to immunohistochemical staining, heat-based antigen retrieval employing a high pH buffer (Leica, ER2) was performed on all slides. As a secondary system, a polymeric detection kit (Refine, Leica) was used. PLAG1 immunopositivity was detected in the nuclei of tumor cells. Both ductal and myoepithelial cells showed positive PLAG1 staining but the immunopositivity was more prevalent in the myoepithelial cells. A tumor was considered as positive for PLAG1 IHC when nuclear immunostain was noted in > 5% of tumor cells.

PLAG1 fluorescence in situ hybridization (FISH)

FISH on interphase nuclei from paraffin-embedded 4-μm sections was performed using custom probes of bacterial artificial chromosomes (BACs) flanking *PLAG1* on chromosome

8q12 (Supplementary Table 1) was performed in 44 cases as previously described [2]. These 44 cases included 15 PA, 12 CAs ex-PA, 3 de novo SDCs, six de novo MECAs, two basal cell adenocarcinoma, and six PACs.

Two hundred successive nuclei were examined for the presence of *PLAG1* gene rearrangements/amplifications using a Zeiss fluorescence microscope (Zeiss Axioplan, Oberkochen, Germany), controlled by Isis 5 software (Metasystems, Waltham, MA). A positive FISH score was interpreted when at least 20% of the nuclei showed a break-apart signal. Nuclei with incomplete set of signals were omitted from the score.

Statistical analysis

All statistical analyses were performed using the SPSS software 22.0 (IBM Corporation, New York, NY, U.S.). The frequency of tumors with PLAG1 immunopositivity as well as the percentage of positive tumor cells in different tumor types was compared using Fisher's exact test and two-tailed student t test respectively. P values less than 0.05 were considered to be statistically significant.

RESULTS

The results of PLAG1 IHC and *PLAG1* FISH are shown in Table 1. PLAG1 IHC was positive in 22 out of 23 (96%) PAs (Figure 1 A-C) and nine out of 15 (60%) CAs ex-PA (Figure 2 A-D). The sensitivity of PLAG1 immunostaining in predicting PA and CA ex-PA were 96% and 60%, respectively. Five out of nine (56%) SDCs ex-PA were positive for PLAG1 IHC, while all six tested de novo SDCs were negative. PLAG1 IHC could differentiate CA ex-PA from de novo SDC (Fisher's exact test, $p = 0.020$). Three out of three (100%) MECAs ex-PA and seven out of ten (70%) de novo MECAs were positive for PLAG1 IHC (Figure 2 E-F). PLAG1 was not a useful marker to distinguish CA ex-PA from de novo MECA (Fisher's exact test, $p = 0.601$).

Among the tumors in the control group, 11 out of 45 tumors demonstrated positive PLAG1 immunostaining, showing a specificity of PLAG1 IHC in predicting the PA component of 74%. The histologic types of these 11 tumors were as follow: seven out of ten (70%) de novo MECAs, two out of four (50%) basal cell adenocarcinomas, one out of two (50%) EMCs, and one out of four (25%) MECs. None of the de novo SDCs (0/6), PACs (0/8), ACCs (0/5), AciCCs (0/5), or secretory carcinomas (0/1) showed positive staining for PLAG1.

The majority of the PAs (15/23), CAs ex-PA (8/15), and de novo MECAs (6/10) exhibited moderate or strong PLAG1 immunolabeling. The percentage of positive tumor cells for PLAG1 IHC expressed as mean ± standard error of mean were 50±4%, 36±7%, and 65±10% in PAs, CAs ex-PA, and de novo MECAs, respectively. The percentage of positive staining was significantly lower in CA ex-PA compared to PA (two tailed Student t test, $p = 0.008$), while there was no significant difference between de novo MECA and PA ($p = 0.90$), or between MECA and CA ex-PA ($p = 0.07$).

The FISH test was previously performed on 44 cases, and the results were previously reported by our group [2]. The correlation between PLAG1 immunoreactivity and *PLAG1* fusion status is shown in Table 2. Overall, PLAG1 IHC had a sensitivity of 80% and specificity of 59% in predicting *PLAG1* fusion. Three of 15 tumors with proven *PLAG1* fusion on FISH analysis were falsely negative on PLAG1 IHC study. All three cases were CAs ex-PA with the carcinoma component being SDC (n = 2) and carcinoma with squamous and glandular features (or unclassified) (n = 1). Twelve of 30 fusion-negative neoplasms exhibited PLAG1 immunoreactivity, including seven PAs, four de novo MECAS, and one basal cell adenocarcinoma. The specificity of PLAG1 IHC in predicting PLAG1 fusion status was 30% in PA and 100% in CA ex-PA.

DISCUSSION

In 1997, Kas et al. were the first to report *PLAG1-CTNNB1* fusion as the key event in the tumorigenesis of pleomorphic adenoma [3, 27]. Since then, multiple groups have confirmed *PLAG1* rearrangements as the most prevalent molecular event in pleomorphic adenoma and carcinoma ex-pleomorphic adenoma, affecting 24–88% of the tumors [2, 4–8, 27]. In these studies, the *PLAG1* translocation was detected using techniques that might not be readily available in daily pathology practices, e.g. Northern blot analysis [5, 6], RT-PCR [4], or FISH [2, 7, 8, 27]. PLAG1 immunohistochemistry might serve as an accessible and feasible alternative. However, the sensitivity and specificity of PLAG1 IHC in detecting PA and *PLAG1* fusion has not yet been well established.

Fusion involving *PLAG1* locus results in overexpression of PLAG1 oncoprotein, which can be detected by immunohistochemistry [4, 27]. Several recent reports have investigated the utility of PLAG1 IHC as an ancillary tool in diagnosing PA and CA ex-PA in surgical and cytologic specimens. The results of these reports are summarized in Table 3 [4, 20–27]. All but one study have demonstrated that PLAG1 IHC was a highly sensitive marker for PA in surgical specimens, showing positivity in 93% to 100% of PAs. Only one group has reported a low PLAG1 IHC sensitivity of 62% in 22 lacrimal gland PAs, using the commercially available 3B7 PLAG1 monoclonal antibody (Novus Biologicals, Littleton, CO, US) [26]. However, the same group has previously reported 100% PLAG1 immunopositivity in the same cohort using a customized PLAG1 antibody [25]. In cytologic samples, the reported percentage of PLAG1 immunoreactivity in PA was relatively low, being 55 – 73%, which might be in part attributed to different preparation methods and/or storage media that were utilized in cytology.

Compared to PA, the reported PLAG1 IHC immunopositivity appeared to be more variable in CA ex-PA, ranging from 20% to 100% [4, 21, 23, 25–27]. In this study, PLAG1 IHC was positive in 60% of the tested CA ex PA cases. Several mechanisms may explain the wide range of PLAG1 immunopositivity in CA ex-PA. First, many types of salivary gland tumors can show hyalinizing stroma mimicking the PA component of CA ex PA; therefore, some of the reported negative PLAG1 IHC cases of CA ex-PA could have been misclassified. Second, PLAG1 oncoprotein overexpression may be lost during the process of tumor progression or malignant transformation, leading to low PLAG1 expression and

subsequently negative PLAG1 IHC. Taken together, PLAG1 IHC emerges as a highly sensitive marker for PA and but less reliable in detecting CA ex-PA.

The reported specificity of PLAG1 IHC as a marker for PA and CA ex PA seemed to be relatively low. Several previous studies have reported positive PLAG1 IHC in non-PA and non-CA ex-PA tumors (7% and 46%, respectively) [4, 20, 23, 24]. In this study, PLAG1 IHC was positive in 29% of other types of tumors. PLAG1 positive staining has been reported in various salivary gland neoplasms, malignant or benign, including myoepithelioma, basal cell adenoma, MECA, ACC, PAC AciCC, and poorly differentiated basaloid carcinoma [4, 20, 23, 24]. In the present study, PLAG1 IHC was found positive in a majority (7/10, 70%) of de novo MECA carcinoma, despite that none of these tumors showed *PLAG1* gene rearrangements by FISH. This result limits the utility of PLAG IHC in distinguishing de novo MECA from PA which is not always easily done by morphology. Additionally, we found PLAG1 to be positive in a proportion of basal cell adenocarcinoma, EMC, and mucoepidermoid carcinoma, but not in de novo SDC, polymorphous adenocarcinoma, ACC, AciCC, and secretory carcinoma. In our hands, PLAG1 IHC seems to a helpful immunomarker in distinguishing between de novo SDC and CA ex-PA, but not between de novo MECA and CA ex-PA.

Two prior studies have correlated PLAG1 immunoreactivity with PLAG1 fusion status [4, 27]. In one study, using RT-PCR with customized primers for *PLAG1* and PLAG1 IHC, Matsuyama et al. have reported that the sensitivity and specificity of PLAG1 IHC in detecting *PLAG1* fusion were 100% and 0%, respectively. In the second study, Bahrami et al. [27] have reported 67% (12/18) of CA ex-PA with PLAG1 fusion and 77% (17/22) with positive PLAG1 by IHC. The sensitivity and specificity of PLAG1 IHC in detecting *PLAG1* fusion in CA ex-PA were 92% and 17%, respectively. In the present study, the sensitivity and specificity of PLAG1 IHC compared to *PLAG1* fusion were 100% and 30% in PA and 70% and 100% in CA ex-PA. Taken together, it appears that PLAG1 IHC is a sensitive but not specific test in predicting *PLAG1* fusion. A negative PLAG1 IHC might be helpful in excluding *PLAG1* fusion and a PA diagnosis, but a positive PLAG1 IHC may not always predict the existence of *PLAG1* fusion.

CONCLUSIONS

PLAG1 IHC is a sensitive marker and a valuable ancillary test for PA and CA ex-PA, and may point to *PLAG1* gene abnormalities that are characteristics in these tumors. Thus, a negative PLAG1 IHC might be more reliable in excluding a PA diagnosis. However, the reverse remains to be determined if PLAG1 IHC expression in the absence of a positive FISH result is non-specific or implies alternative genetic or epigenetic mechanisms beyond the FISH resolution.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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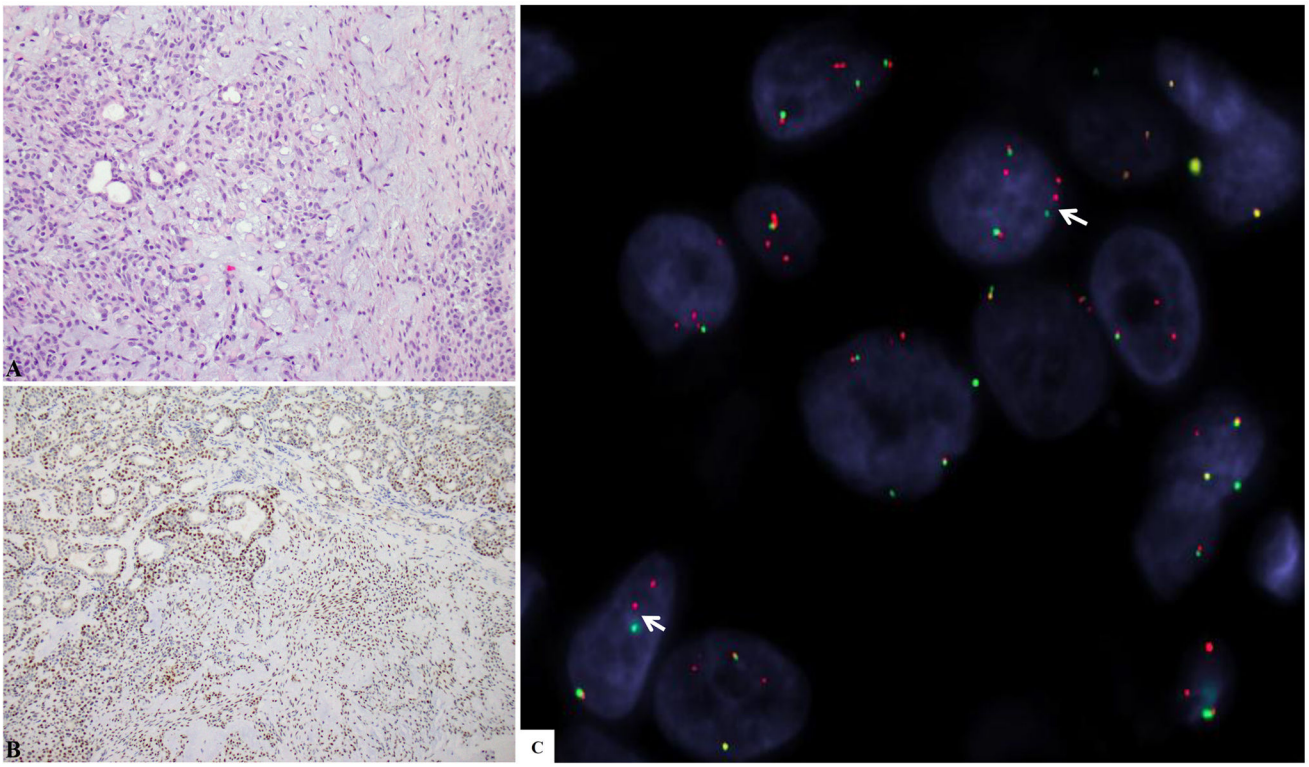

Figure 1.

(A-C): Pleomorphic adenoma (PA). A: H&E stain showing typical histology of PA with ductal structures (epithelial component), myoepithelial cells arranged as cords and nests, and myxoid stroma. B: PLAG1 IHC showing diffuse nuclear labeling. C: FISH for *PLAG1* showing break-apart signal (arrow) (red, centromeric; green, telomeric).

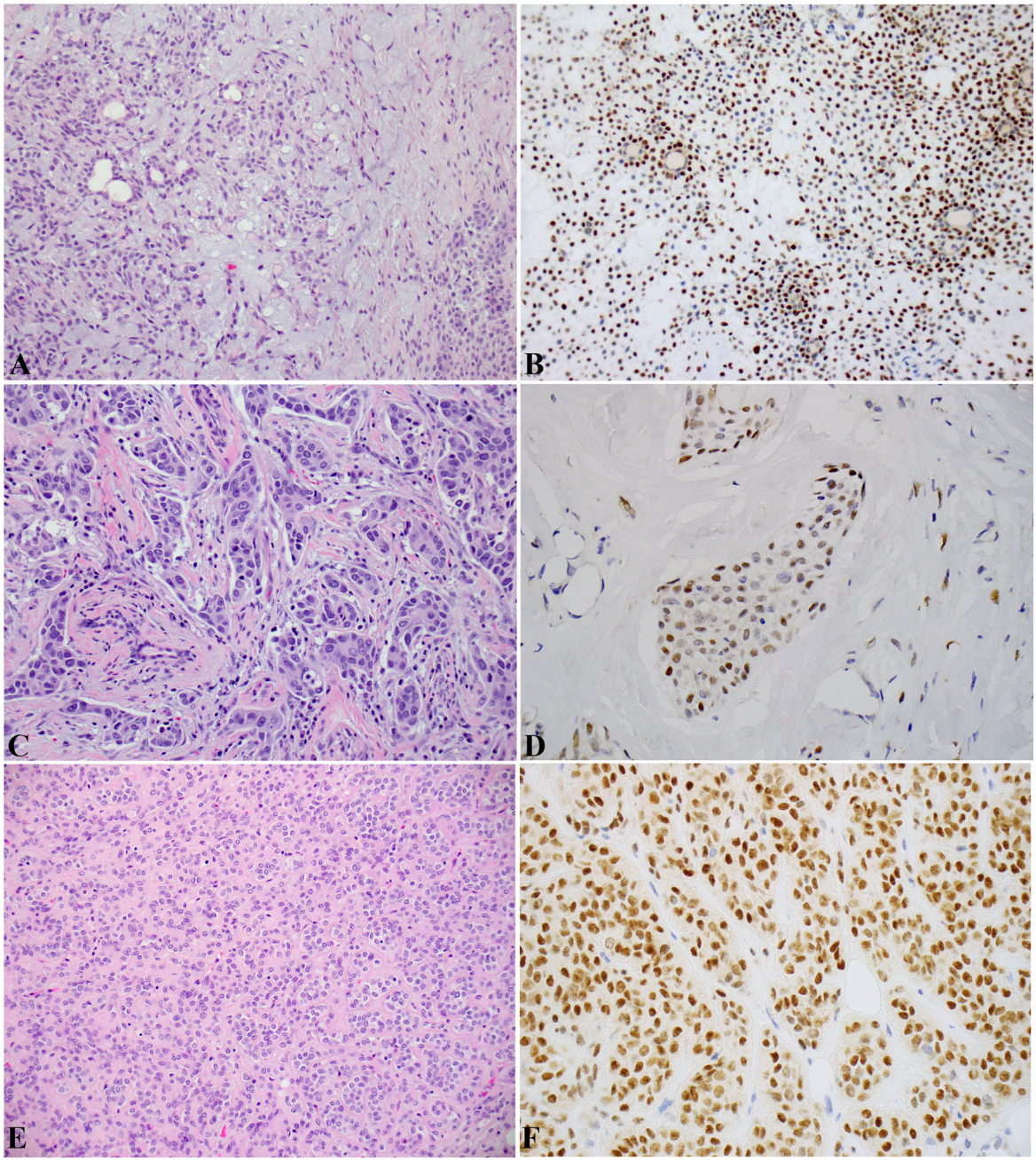

Figure 2.

(A-D): Carcinoma ex-PA (CA ex-PA). A-B: the PA component: histology in (A) and PLAG1 IHC in (B). C-D: the carcinoma component: histology in (C) and PLAG1 IHC in (D). The carcinoma component is a salivary duct carcinoma with typical apocrine cytomorphology and marked nuclear atypia. (E-F): De novo myoepithelial carcinoma: histology in (A) and PLAG1 IHC in (B).

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Table 1

Results of PLAG1 Immunohistochemistry and FISH in salivary gland neoplasms.

	***PLAG1* rearrangements by FISH[a]**	**PLAG1 IHC[a]**	**Percentage of tumor cells positive for PLAG1 (mean ± SEM)**	**PLAG1 intensity in positive cases (N)**		
				Weak	**Moderate**	**Strong**
Pleomorphic adenoma (PA)	5/15 (33%)	22/23 (96%)	50±4%	5	4	11
Carcinoma ex pleomorphic adenoma (CA ex-PA)	10/12 (83%)	9/15 (60%)	36±7%	1	5	3
De novo myoepithelial carcinoma	0/6 (0%)	7/10 (70%)	65±10%	1	1	5
De novo salivary duct carcinoma	0/3 (0%)	0/6 (0%)	NA	NA	NA	NA
Basal cell adenocarcinoma	0/2 (0%)	2/4 (50%)	16±9%	1	1	NA
Polymorphous adenocarcinoma	0/6 (0%)	0/8	NA	NA	NA	NA
Epithelial-myoepithelial carcinoma	ND	1/2 (50%)	50%	NA	NA	1
Mucoepidermoid carcinoma	ND	1/ 4 (25%)	15%	NA	1	NA
Adenoid cystic carcinoma	ND	0/5	NA	NA	NA	NA
Acinic cell carcinoma	ND	0/5	NA	NA	NA	NA
Secretory carcinoma	ND	0/1	NA	NA	NA	NA

[a]Values are expressed as number of positive cases/total number of cases tested (% of positivity).

IHC: immunohistochemistry; FISH: fluorescence in situ hybridization, NA: not applicable, ND: not performed, SEM: standard error of mean N, number of cases tested.

Table 2

Sensitivity and specificity of PLAG1 IHC in predicting *PLAG1* fusion in PA, CA ex-PA and other salivary gland neoplasms.

		PLAG1 IHC (+)	PLAG1 IHC (−)	Sensitivity	Specificity
Overall	*PLAG1* fusion (+)	12	3	80%	59%
	PLAG1 fusion (−)	12	17		
PA	*PLAG1* fusion (+)	5	0	100%	30%
	PLAG1 fusion (−)	7	3		
CA ex-PA	*PLAG1* fusion (+)	7	3	70%	100%
	PLAG1 fusion (−)	0	2		
Others	*PLAG1* fusion (+)	0	0	NA	71%
	PLAG1 fusion (−)	5	12		

Table 3

A comprehensive literature review of PLAG1 immunohistochemistry in PA, Ca ex-PA and other salivary gland tumors.

Ref.	Tumors tested (n)	Antibody clone	Criteria for PLAG1 IHC positivity	PLAG1 immunopositivity in PA/CA ex-PA	PLAG1 immunopositivity in non-PA	*PLAG1* Fusion, detecting technique	Sensitivity and specificity of PLAG1 IHC in detecting PLAG1 fusion in PA
PLAG1 IHC in pleomorphic adenoma (PA) (surgical specimens)							
a	PA (23) Non-PA (45)	Novus 3B7	> 5% positive cells	22/23 (96%)	13/45 (29%)	5/15 (33%), FISH	Sensitivity: 100% Specificity: 30%
4	PA (45) Non-PA (42)	Sigma-Aldrich Co., 1:500	NA	45/45 (100%)	3/42 (7%)	11/45 (24%), RT-PCR	Sensitivity 100% Specificity 0%
20	PA (36) Non-PA (37)	Novus 3B7 1:20	Nuclear staining > 5 (intensity X %)	33/36 (92%)	17/37 (46%)	NA	NA
21	PA (40)	Novus 3B7 1:100	> 10% positive cells	37/40 (93%)	NA	NA	NA
22*b*	PA (76)	Novus 3B7 1:100	> 10% positive cells	71/76 (94%)	NA	NA	NA
23	PA (36) Non-PA (64)	Novus 3B7 1:25	Any nuclear staining	34/36 (94%)	1110/64 (16%)	NA	NA
25, 26	PA (26)	Astrom et al. 2000 [16]	Any nuclear staining	26/26 (100%)	NA	NA	NA
	PA (28)	Novus 3B7	NA	16/26 (62%)	NA	NA	NA
PLAG1 IHC in pleomorphic adenoma (PA) (cytologic specimens)							
24	PA (30) Non-PA (39)	Novus 3B7 1:100	Moderate to strong nuclear staining	22/30 (73%)	4/39 (10%)	NA	NA
20	PA (40) Non-PA (12)	Novus 3B7 1:20	Nuclear staining > 5 (intensity X %)	22/40 (55%)	3/12 (25%)	NA	NA
PLAG1 IHC in carcinoma ex pleomorphic adenoma							
a	CA ex-PA	Novus 3B7 1:20	> 5% positive cells	9/15 (60%)	NA	12/14 (86%) FISH	Sensitivity: 70% Specificity: 100%
27	CA ex-PA (22) Non-PA (39)	Novus 3B7	>50% positive cells	17/22 (77%)	NA	12/18 (67%), FISH	Sensitivity: 92% Specificity: 17%
4	CA ex-PA (4)	Sigma-Aldrich Co., 1:500	NA	4/4 (100%)	NA	0/4 (0%) RT-PCR	Sensitivity: NA Specificity: 0%
23	CA ex-PA (1)	Novus 3B7 1:25	Any nuclear staining	1/1 (100%)	NA	NA	NA
21	CA ex-PA (40)	Novus 3B7 1:100	> 10% positive cells	14/40 (35%)	NA	NA	NA
25, 26	CA ex-PA (5)	Novus 3B7	NA	1/5 (20%)	NA	NA	NA
	CA ex-PA (5)	Astrom et al 2000 [16]	Any nuclear staining	3/5 (60%)	NA	NA	NA

[a] Data from the present study.

[b] These 76 patients included the 40 patients studied in the above row.

NA: Not available, RT-PCR: reverse-transcription polymerase chain reaction.