

***PLAG1* Alteration in Carcinoma Ex Pleomorphic Adenoma: Immunohistochemical and Fluorescence In Situ Hybridization Studies of 22 Cases**

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Received: 30 January 2012 / Accepted: 26 March 2012 / Published online: 8 April 2012
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Abstract Carcinoma ex pleomorphic adenoma (CA-ex-PA) may arise with nearly any histologic subtype of carcinoma of the salivary gland. In the absence of recognizable residual pleomorphic adenoma (PA) or a prior history of PA, distinction of CA-ex-PA from morphologically similar de novo carcinomas may be difficult. Oncogenic rearrangement of *PLAG1* (*pleomorphic adenoma gene 1*) has been established in PA; however, it has not yet been proven that *PLAG1* alteration persists in carcinomas developed from preceding PA. We evaluated 22 histologically diverse CA-ex-PA by immunohistochemistry for *PLAG1*, and/or by FISH targeting *PLAG1*. Of these, 17 cases were immunoreactive (1+ to 3+) and 5 were immunonegative/rare positive for *PLAG1*. For comparison, 39 various salivary gland neoplasms were immunostained for *PLAG1*, of which all scored negative/rare positive. Twelve of 19 CA-ex-PA analyzed by *PLAG1* FISH (63 %) were positive for gene rearrangement, 2 showed only a trisomy/polysomy profile, and 5 had a normal pattern. One FISH-positive tumor showed amplification of *PLAG1*. One of 3 cases analyzed for *HMGA2* FISH was positive for gene rearrangement. In our series, the majority of CA-ex-PA harbored altered *PLAG1* or *HMGA2* genes detectable by FISH. While *PLAG1* immunostain was specific for CA-ex-PA against other carcinomas, its application as a standalone discriminatory test was limited by variable expression. We conclude that most CA-ex-PA, regardless of morphologic

subtype, carry altered *PLAG1* or *HMGA2* genes, and that FISH for *PLAG1*, along with immunohistochemistry for *PLAG1*, may help discriminate CA-ex-PA from its de novo carcinoma counterpart.

Keywords Carcinoma ex pleomorphic adenoma · *PLAG1* · Fluorescence in situ hybridization · Immunohistochemistry · Salivary gland

Introduction

The oncogenic effect of recurrent chromosomal translocations in leukemias and sarcomas has been well established over the past few decades. In contrast, in epithelial neoplasms, the role of fusion transcripts has only recently gained recognition. This difference may be attributable to the karyotypic complexity of many carcinomas and the failure of traditional cytogenetic techniques to find putative fusion genes amidst other genetic imbalances accumulated during tumor progression. Recently, recurrent chimeric genes have been described in a number of salivary gland malignancies, including mucoepidermoid, hyalinizing clear cell, and adenoid cystic carcinomas, adding to the small group of translocation-associated carcinomas known to date [1, 2]. Carcinoma ex pleomorphic adenoma (CA-ex-PA), however, is unique among both salivary gland tumors and other epithelial malignancies for its association with a benign preexisting lesion whose genetic characteristics have already been well described.

Conventional cytogenetic analysis has detected recurrent translocations in approximately 70 % of pleomorphic adenomas (PA) [3–6]. These translocations target primarily *PLAG1* (*pleomorphic adenoma gene 1*), resulting in upregulation of this gene, located at 8q12, and overexpression of

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its protein (PLAG1) [7, 8]. The second, much less frequent target gene in PA is *HMGA2*, mapped to 12q14-15 [9, 10]. The pathogenic role of PLAG1, however, appears to span all PA, including tumors without cytogenetic evidence of *PLAG1* alteration and those with *HMGA2* rearrangement. This conclusion is drawn from immunohistochemical and Northern blot studies showing PLAG1 overexpression in nearly all PA, regardless of *PLAG1* status [11–13].

Few CA-ex-PA have been evaluated for *PLAG1* status [11, 12, 14–16]. Martins et al. [15] found *PLAG1* rearrangement in 3 cases and trisomy 8 without gene alteration in 1 case among 4 selected CA-ex-PA that were known to have karyotypic deviations of chromosome 8 [15]. On the other hand, Matsuyama et al. [12] conducted RT-PCR assays for the classic fusion transcripts of PA in 4 CA-ex-PA and found none of them to harbor chimeric genes of interest [12]. Resolving this issue is relevant from both biologic and diagnostic standpoints. CA-ex-PA is histologically diverse with malignant components that can resemble distinct types of salivary gland carcinomas or variable admixtures of different tumor types including heterologous mesenchymal elements in some instances. At clinical presentation, the malignant component of CA-ex-PA often overruns the preexisting PA and the presence of a precursor PA may only be suggested by extensive areas of tumor hyalinization. Moreover, the histologic diversity of CA-ex-PA increases the likelihood of misclassification, both as other salivary and non-salivary malignancies, in small biopsy specimens. Therefore, defining a specific immunohistochemical or genetic marker for CA-ex-PA has practical diagnostic implications. With these thoughts, we conducted the present study with the following objectives: first, to determine the status of *PLAG1* rearrangement and overexpression of PLAG1 protein in the carcinomatous component of PA, and second, to discern the validity of PLAG1 protein as a potential diagnostic marker for CA-ex-PA versus other carcinoma types.

Materials and Methods

Materials

This study was approved by the respective institutional review boards at both participating institutions. All tumors were defined per WHO criteria for salivary gland neoplasms [17]. Twenty-two histologically confirmed CA-ex-PA with sufficient material for the study were retrieved from the pathology archives at Brigham and Women's Hospital. Of these, 20 cases were primary CA-ex-PA (including 4 non-invasive and 1 minimally invasive) and 2

cases were metastatic tumors. All study subjects had either a pre-existing PA adjacent to the carcinoma or a history of prior excision for a PA at the same location (see Table 1). Metastatic tumors were confirmed as being histologically similar to the corresponding CA-ex-PA at the presumed primary site.

For comparison, 39 cases of various benign and malignant salivary gland neoplasms among the following histologic categories were randomly selected: adenocarcinoma, NOS (4 cases), mucoepidermoid carcinoma (8 cases), adenoid cystic carcinoma (7 cases), salivary duct carcinoma (4 cases), epithelial-myoeplithelial carcinoma (2 cases), polymorphous low-grade adenocarcinoma (1 case), myoeplithelial carcinoma (1 case), acinic cell carcinoma (1 case), basaloid carcinoma (1 case), pleomorphic adenoma (5 cases), basal cell adenoma (4 cases), and low-grade salivary gland neoplasm, NOS (1 case). In addition, tissue microarray sections of a diverse group of carcinomas, comprising ductal carcinoma of breast (46 samples), gastric adenocarcinoma (10), esophageal squamous carcinoma (9), lung adenocarcinoma (10), colorectal adenocarcinoma (10), papillary thyroid carcinoma (10), and renal cell carcinoma (9), were evaluated to assess PLAG1 immunoreactivity in other carcinoma types.

Immunohistochemical Study

Formalin-fixed, paraffin-embedded tissue blocks from 61 study and control cases were cut into 4 μm -thick sections and immunostained with a monoclonal PLAG1 antibody (clone 3B7, 1:50; Novus, Littleton, CO), using heat-induced epitope retrieval (in citrate buffer for 30 min) and the bond polymer refine detection system (Leica Microsystems, Bannockburn, IL) after 2 h of antibody incubation at room temperature. Positive controls were included in each run, using sections of a classic PA or a lipoblastoma. Immunostains were semiquantitatively scored as follows: No positive cells, "negative"; 1–4 % positive cells, "rare"; 5–25 % positive cells, "1+"; 26–50 % positive cells, "2+"; >50 % positive cells, "3+". The intensity of staining was graded as weak, intermediate, and strong. Only the carcinomatous component was scored and graded for PLAG1 immunoreactivity in CA-ex-PA subjects.

Fluorescence In Situ Hybridization Study

Break-apart, dual-color FISH probes for *PLAG1* were developed using bacterial artificial chromosome (BAC) probes including the RP11-22E14 clone, flanking the 3' end, and the RP11-1130K23 clone, flanking the 5' end of

Table 1 Demographics and histologic characteristics of 22 cases of carcinoma ex pleomorphic adenoma

Case #	Age/ sex	Location	Histologic findings	Grade	PLAG1 IHC (score/intensity)	PLAG1 FISH	HMGA2 FISH
1. (Fig. 1-1)	29 M	Lt buccal (recurrent)*	Adeno-CA, NOS (recurrent)	L	1+/strong	Pos	NP
2. (Fig. 1-2)	86 M	Parotid	Myoepithelial CA	I	3+/strong	Pos	NP
3. (Fig. 1-3)	66 M	Lt submandibular	Epithelial- myoepithelial CA	I	3+/strong	Pos (unbal translocation) with polysomy	NP
4. (Fig. 1-4)	51/53 F	Lt parotid**	Adeno-CA	I	2+to 3+/intermediate to strong	Neg with polysomy (primary, 3–4 copies; recurrence, 3–8 copies)	NP
5. (Fig. 2-1)	78 F	Lung met*	Adeno-CA, NOS	H	2+/weak	Pos with trisomy	NP
6. (Fig. 2-2)	58 M	Liver met***	Salivary duct CA	H	3+/intermediate	Pos (multi-allelic translocation) with polysomy	NP
7. (Fig. 3)	55 M	R parotid	Salivary duct, adeno-CA, myoepithelial CA	H	Rare/weak	Neg	Pos
8.	60 F	R parotid	Myoepithelial CA	I	2+/strong	Pos (bal & unbal translocation) with trisomy	NP
9.	78 F	R parotid	Myoepithelial CA	L	3+/intermediate	Pos	NP
10.	84 F	Lt parotid	Myoepithelial CA, minimally invasive	L	1+/weak	NP	NP
11.	51 F	R parapharynx	Epithelial- myoepithelial CA	I	2+/intermediate to strong	Neg	NP
12.	42 F	Lt submandibular	Adeno-CA, NOS (recurrent)	L	2+/intermediate to strong	Pos (bi-allelic translocation) with trisomy	NP
13.	53 M	Lt parotid	Adeno-CA, NOS	I	Negative	Pos	NP
14.	58 F	R parotid	Carcinoma and sarcoma	I	2+/intermediate	Neg	Neg
15.	44 M	R parotid	Salivary duct, squamous cell CA	H	Negative	NP	NP
16.	63 M	R parotid	Salivary duct CA	H	Rare/intermediate	NP	NP
17.	63 M	Lt submandibular	Salivary duct CA	H	Negative	Neg	Neg
18.	24 M	R parotid	Myoepithelial, salivary duct CA	H	1+/intermediate	Pos (unbal translocation) with <i>PLAG1</i> amplification	NP
19.	54 F	Parapharynx	Adeno-CA, NOS, multifocal, non- invasive	H	1+/strong	Neg with trisomy in >50 % of cells	NP
20.	81 M	R parotid	Adeno-CA, NOS, non-invasive	H	3+/intermediate to strong	Pos (unbal translocation) with trisomy	NP
21.	62 M	R submandibular	Salivary duct, mucinous CA, non-invasive	H	3+/strong	Neg	NP
22.	44 M	R oropharynx	Squamous cell CA, non-invasive	I	3+/strong	Pos	NP

bal balanced translocation pattern, *CA* carcinoma, *Chr* chromosomem, *H* high, *I* intermediate, *L* low, *Lt* left, *met* metastasis, *Neg* negative for gene rearrangement, *NP* not performed, *Pos* positive for gene rearrangement, *R* right, *unbal* unbalanced translocation pattern

* Left parotid primary; ** samples from both original and recurrent tumors; *** right submandibular primary

the *PLAG1* gene. FISH assay for *HMGA2* was developed using BAC probes to include the RP11-662G15 clone, flanking the 5' end, and the RP11-1025D9 clone, flanking the 3' end. BAC DNA was isolated using a modified

Qiagen plasmid extraction protocol. The RP11-22E14 sequence was labeled with AlexaFluor 488 (green fluorochrome), and the RP11-1130K23 sequence with Rhodamine (red fluorochrome). The RP11-662G15 sequence

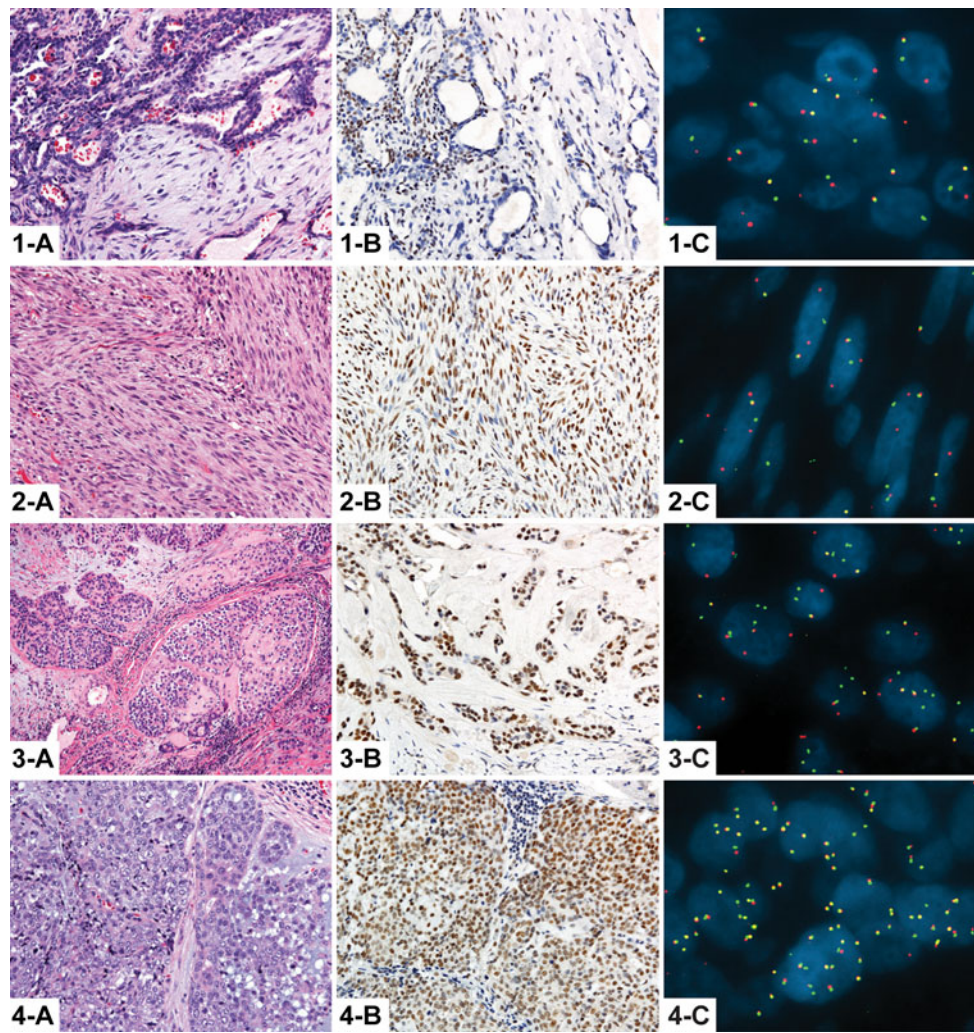


Fig. 1 H&E microscopy, immunohistochemistry for *PLAG1*, and fluorescence in situ hybridization study for *PLAG1* in 4 cases of carcinoma ex pleomorphic adenoma (CA-ex-PA) of the salivary gland. Low-grade invasive CA-ex-PA with histologic appearance of adenocarcinoma NOS (**1-A**), showing variable *PLAG1* expression with strong intensity (**1-B**). FISH for *PLAG1* shows one pair of split signals indicative of *PLAG1* rearrangement with a balanced translocation pattern (**1-C**). Intermediate-grade myoepithelial CA-ex-PA with spindle cell morphology (**2-A**), showing diffuse expression of

PLAG1 (**2-B**), and *PLAG1* rearrangement with additional copies of red signal, corresponding to the 5' end of *PLAG1* (**2-C**). Intermediate-grade epithelial-myoepithelial cell CA-ex-PA (**3-A**), with a diffuse expression of *PLAG1* (**3-B**). FISH demonstrates one or more copies of rearranged *PLAG1* with an unbalanced translocation pattern and a polysomy profile (**3-C**). Intermediate-grade recurrent adenocarcinoma ex pleomorphic adenoma (**4-A**), showing strong immunoreactivity for *PLAG1* (**4-B**). FISH for *PLAG1* reveals a polysomy pattern with up to 8 copies of the intact gene (**4-C**)

was labeled with the green and the RP11-1025D9 sequence with the red fluorochrome. Dual-color FISH was performed on 4 micron-thick FFPE tissue sections, as previously described [18]. For CA-ex-PA cases, a section comprising the largest available carcinomatous component was selected. A normal control produced two joined green and red (yellow) signals on chromosome 8 in each nucleus.

Material was available to perform FISH for *PLAG1* in 19 of 22 CA-ex-PA, and in all 5 PA. Subsequently, FISH for *HMGA2* was conducted in CA-ex-PA subjects that were found to be negative for *PLAG1* rearrangement by FISH (when adequate material was available).

Results

Clinicopathologic Findings

The demographics and pathologic findings of the 22 study cases are presented in Table 1. In summary, CA-ex-PA occurred in 13 males and 9 females, ranging from 24 to 86 years old (median, 58 years old), and involved the following salivary glands/anatomic locations: parotid gland (13 cases), submandibular gland (4 cases), parapharynx (2 cases), oropharynx (1 case), and metastatic sites (2 cases, 1 each in liver and lung). The malignant component comprised one or more of these histologic subtypes:

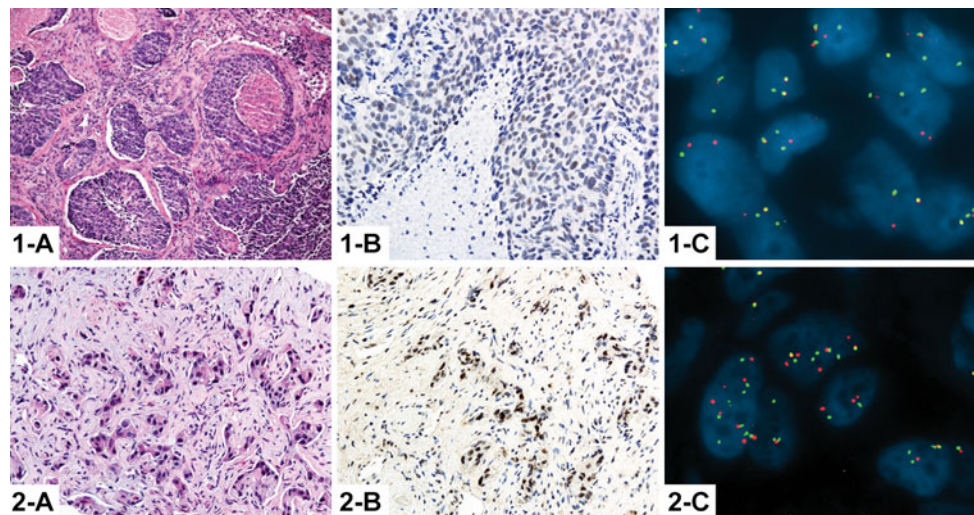


Fig. 2 H&E microscopy, immunohistochemistry for *PLAG1*, and FISH for *PLAG1* in 2 cases of metastatic CA-ex-PA. High-grade metastatic adenocarcinoma ex pleomorphic adenoma to the lung (1-A), with weak nuclear expression of *PLAG1* (1-B). FISH

demonstrates 1–2 copies of rearranged *PLAG1* and a trisomy profile (1-C). High-grade metastatic salivary duct CA-ex-PA to the liver (2-A) with nuclear expression of *PLAG1* (2-B). FISH shows a polysomy pattern and multiple copies of rearranged *PLAG1* (2-C)

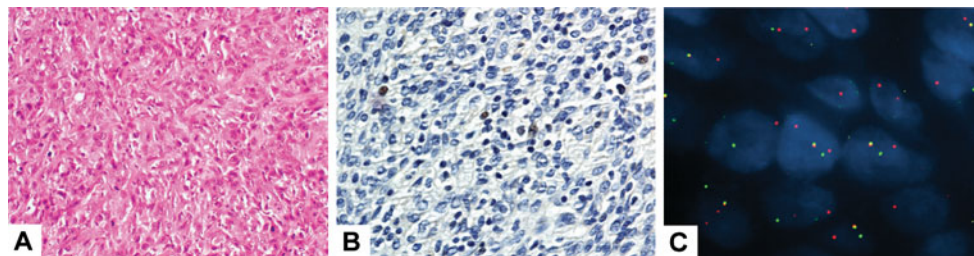


Fig. 3 H&E microscopy, immunohistochemistry for *PLAG1*, and FISH for *HMGA2* in a high-grade primary CA-ex-PA comprising various histologic subtypes (adenocarcinoma, myoepithelial and

salivary duct carcinomas) (A), with rare *PLAG1* positive cells (B). FISH for *PLAG1* had a normal pattern (not shown). FISH for *HMGA2* demonstrates one pair of split signals reflective of gene alteration (C)

adenocarcinoma, NOS (7 cases), myoepithelial carcinoma (4 cases), salivary duct carcinoma (3 cases), epithelial-myoepithelial carcinoma (2 cases), squamous cell carcinoma (1 case), carcinosarcoma (1 case), and mixed histology with a combination of 2 or more of adenocarcinoma NOS, salivary duct, myoepithelial, mucinous, and squamous cell carcinoma (4 cases). Ten CA-ex-PA were high-grade, 8 were intermediate-grade, and 4 were low-grade.

Immunohistochemical Findings

Of 22 CA-ex-PA examined for *PLAG1* expression, 7 cases scored “3+”, 6 cases scored “2+”, 4 cases scored “1+”, 2 cases scored “rare”, and 3 cases scored “negative” (Table 1). Of 5 pleomorphic adenomas, 3 cases scored “3+” and 2 cases scored “2+”. The intensity of staining was variable, and sometimes weak. All other salivary gland neoplasms scored “rare/negative” for *PLAG1* immunostain. Tissue microarray sections of carcinomas from

various organs were negative in all but 1 infiltrating ductal carcinoma, which was positive for *PLAG1* in rare cells.

Fluorescence In Situ Hybridization Study Findings

The details of FISH findings are presented in Table 1. In summary, of 19 CA-ex-PA analyzed by *PLAG1* FISH, 12 cases were positive for gene rearrangement with a balanced or an unbalanced translocation pattern, two cases showed a trisomy/polysomy pattern without gene rearrangement (increased copies of an intact *PLAG1*), and 5 had a normal FISH pattern. In tumors positive for *PLAG1* alteration, additional chromosomal abnormalities were found, including a trisomy/polysomy profile (6 cases), and amplification of *PLAG1* (1 case). The gene rearrangement could be seen in one or both genes (one to two pairs of split signals in a cell), or in multiple genes (rearrangement in the setting of polysomy). In cases positive for *PLAG1* alteration, split signals were seen in 56–95 % of examined

cells. Of 3 cases analyzed for *HMGA2* FISH, 1 case was positive for gene rearrangement (in 94 % of cells analyzed). Eleven of 12 FISH positive cases expressed *PLAG1* by immunohistochemistry (1+ to 3+ ; weak to strong), and 1 case was negative (on one representative section). Of the 5 PA, FISH was positive for gene rearrangement with a balanced translocation pattern in 3 cases, negative in 1 case, and failed in 1 case on multiple attempts due to poor hybridization.

Discussion

Carcinomatous transformation is an uncommon but well-known risk associated with PA (referred to as CA-ex-PA). It accounts for <4 % of all salivary gland neoplasms, and <12 % of malignant salivary gland tumors [17]. CA-ex-PA may develop with a wide-spectrum of histologic subtypes; however, adenocarcinoma NOS, salivary duct carcinoma, or myoepithelial carcinoma [19–21] occurs most commonly.

The diagnosis of CA-ex-PA requires recognition of a co-existing component of PA, or a history of prior excision of a PA at the same site. However, the malignant component frequently takes over the entire tumor; as a result, the underlying PA may no longer be identifiable. According to one account, examination of up to 100 cut sections is sometimes needed to find traces of small residual pre-existing PA [22]. While having multiple carcinoma subtypes in a given mass or marked stromal hyalinization strongly suggests the possibility of CA-ex-PA, these features are not sufficiently specific for a definitive diagnosis. A reliable marker for CA-ex-PA, therefore, would be a valuable ancillary tool in cases of diagnostic uncertainty. In the salivary gland, it is important to be able to distinguish CA-ex-PA from metastatic disease. Conversely, at metastatic sites, it is important to be able to distinguish metastatic CA-ex-PA from other potential primary tumors. With the prospect of increasingly targeted cancer therapies, distinction of CA-ex-PA from other clinically aggressive salivary gland malignancies is also likely to take on greater significance.

Based on the current state of knowledge, translocations involving *PLAG1* are known to be specific for two pathologic entities, PA and lipoblastoma. In addition, we have recently demonstrated *PLAG1* alteration in a subset of mixed tumors of the skin and soft tissue [18]. The *PLAG1* gene produces a zinc finger protein with roles during embryogenesis and fetal development. In adult tissues, on the other hand, the protein is either undetectable or present at negligible levels [23, 24]. Translocations involving *PLAG1* lead to upregulation of the gene and ectopic production of *PLAG1* protein mediated by promoter swapping

between *PLAG1* and a ubiquitously expressed partner gene [13, 23, 24]. The preferential partner gene in almost half of PA is the gene for β -catenin (*CTNNB1*) [23]. The second most common participating gene is the leukemia inhibitory factor receptor (*LIFR*) gene [25]. The molecular mechanisms of *PLAG1* alteration in lipoblastoma are similar to those in PA but involve different partner genes, *COL1A2* or *HAS2* [26, 27].

PLAG1 protein expression, on the other hand, has been seen in other tumors including a subset of acute myeloid leukemia [28], hepatoblastoma [29], and a variety of mesenchymal tumors [11, 30]. *PLAG1* overexpression also encompasses PA without cytogenetic finding of *PLAG1* alteration, including those with *HMGA2* rearrangement and karyotypically normal tumors [11, 12]. Cryptic intra-chromosomal rearrangements, not detectable by routine cytogenetic studies, involving two genes close to *PLAG1*, namely *TCEA1* and *CHCHD7*, account for a subset of these tumors [11, 31]. Other mechanisms, such as mutational activation or copy number gain of *PLAG1*, have been postulated in *PLAG1* overexpression [11, 32]. Numerical gain of chromosome 8 or increased copy number of intact *PLAG1*, as an isolated anomaly, was seen in 2 subjects in our series, although the mechanism is unclear through which increased dosage of a putatively inactive gene ultimately leads to protein overexpression.

The findings from our study support that *PLAG1* is a specific marker for CA-ex-PA among other carcinomas, both those primary to the salivary gland and a variety of other common carcinomas from other primary sites. Outside the salivary gland, one should also consider the uncommon possibility of malignant mixed tumors of skin/soft tissue. Obviously neither FISH nor immunohistochemistry can help to discriminate between a malignant mixed tumor of soft tissue and a metastatic CA-ex-PA, and clinical history would be the most helpful clue in this situation.

While *PLAG1* was not an entirely robust immunostain for CA-ex-PA in our series, we found it a useful adjunctive marker, especially when interpreted in conjunction with *PLAG1* FISH findings. The utility of *PLAG1* immunostaining was limited in some cases by variable, focal, or weak staining. In our experience, however, a diffuse and unequivocal expression for *PLAG1* in a carcinoma is a strong indication for the diagnosis of CA-ex-PA in the proper clinicopathologic setting. One case in our series, despite cytogenetic confirmation of *PLAG1* rearrangement, failed to show *PLAG1* immunoreactivity, and 4 additional pathologically confirmed CA-ex-PA were also negative. Since some of our samples were retrieved among old archival material, we cannot exclude that the mode of tissue fixation had not contributed to the lack of protein expression in some cases.

The combination of immunohistochemistry and FISH for *PLAG1*, with or without FISH for *HMGA2*, appears to be a more practical approach in investigating the possibility of CA-ex-PA compared to RT-PCR, in view of the multiplicity of *PLAG1* partner genes. Matsuyama et al. [12] performed RT-PCR on 45 PA, screening for multiple known fusion genes with the following positive results: *CTNNB1-PLAG1* (8 cases), *LIFR-PLAG1* (2 cases), and one each for *CHCHD7-PLAG1* and *HMGA2-WIF1*. None of the cases had *TCEA1-PLAG1*, *HMGA2-FHIT*, and *HMGA2-NFIB* fusion transcripts. In our series, 63 % of CA-ex-PA had *PLAG1* rearrangement, closely matching the frequency of translocations in PA observed by cytogenetic studies (nearly 70 %). Considering that alterations of *PLAG1* and *HMGA2*, and a trisomy/polysomy profile for chromosome 8 (or gain of intact *PLAG1*) are the relevant genetic alterations in PA, only 4 (21 %) of CA-ex-PA in our series had a normal pattern for *PLAG1* or *HMGA2* FISH assays. It is notable that cryptic intrachromosomal *PLAG1* rearrangements are likely undetectable by FISH; a normal FISH pattern, therefore, does not entirely exclude the possibility of gene rearrangement.

The pathogenesis and genetic events in malignant progression of PA are not entirely understood. The risk is likely increased with the duration of the mass [19, 33]; therefore, it is conceivable that as a tumor grows, acquisition of additional genetic anomalies leads to malignant progression. Marked numerical and structural chromosomal aberrations and DNA aneuploidy are found in CA-ex-PA, but which, if any, of these changes are crucial in malignant transformation is yet to be determined [34, 35]. A study by El-Naggar et al. [35] suggested that LOH in the 12q region identifies a group of PA with a potential for evolution to carcinoma, and that additional changes at 17p may be the preceding events for malignant transformation [36]. Using a genome-wide, high-resolution array-CGH, Persson et al. [37] found amplification of 12q genes (specifically *MDM2*), deletions of 5q23.2-q31.2, gains of *PLAG1* and *MYC*, and amplification of *ERBB2* to be relevant events in malignant change [37]. Tsang et al. [16] reported a case of CA-ex-PA with amplification of multiple genes including *PLAG1* [16]. We have also recently demonstrated increased copy number and amplification of rearranged *PLAG1* in a CA-ex-PA in a young patient who had a history of PA with simple *PLAG1* rearrangement [38]. An additional case in our current series showed amplification of altered *PLAG1*. In addition, by FISH, six of CA-ex-PA in our study had *PLAG1* alteration with a trisomy/polysomy pattern. Our findings, in addition to previous reports, suggest that copy number gain and amplification of *PLAG1* may contribute to malignant progression in a subset of tumors. Amplification and overexpression of *HMGA2* has also been postulated in

transformation to CA-ex-PA [39]. It seems more likely that malignant degeneration may be triggered by a variety of mechanisms, rather than a single genetic event responsible for all tumors. The role of p53 mutation in malignant transformation of PA, however, has already been looked at, and refuted by mutational analysis of 11 cases [40].

In summary, we evaluated 22 cases of CA-ex-PA by immunohistochemistry for *PLAG1* and by FISH targeting *PLAG1*. In our series, the majority of CA-ex-PA carried an altered *PLAG1* gene that could be identified by FISH technique. We conclude that *PLAG1* rearrangement persists in carcinomas arising from PA, confirming the few previous observations. *PLAG1* expression in CA-ex-PA was variable but was specific against other salivary gland carcinomas and carcinomas of other primary sites when staining was robust. In situations where there is a question of malignancy arising in the context of preexisting PA, FISH for *PLAG1* and immunohistochemistry for *PLAG1*, particularly in combination, are helpful ancillary studies to facilitate diagnostic interpretation.

Acknowledgments This work was supported in part by the American Lebanese Syrian Associated Charities (ALSAC). The study was performed with the approval of the Institutional Review Boards of St. Jude Children's Research Hospital and Partners Health Care. IRB numbers: NR10-130 and 2010-P-001957/1.

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