Clinicopathological effect of \textit{PLAG1} fusion genes in pleomorphic adenoma and carcinoma ex pleomorphic adenoma with special emphasis on histological features

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Short title: Clinicopathological impacts of PLAG1 fusion in PA

Keywords: Pleomorphic adenoma, carcinoma ex pleomorphic adenoma, \textit{PLAG1}, \textit{HMGA2}, RT–PCR

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\textit{Aims}: Pleomorphic adenoma gene 1 (\textit{PLAG1}) rearrangement is well known in pleomorphic adenoma (PA), which is histologically characterised by admixed epithelial and mesenchymal components. Multiple fusion variants of \textit{PLAG1} and \textit{HMGA2} have been reported; currently, however, little is known regarding the clinicopathological impacts of these fusion types

\textit{Methods and results}: We examined the \textit{PLAG1}- and \textit{HMGA2}-related fusion status in 105 PAs and 11 cases of carcinoma ex PAs (CXPA) arising from salivary glands and lacrimal glands to elucidate their
correlation to the clinicopathological factors. Forty cases harboured *PLAG1* fusion genes: *CTNNB1–PLAG1* in 22 cases, *CHCHD7–PLAG1* in 14 cases and *LIFR–PLAG1* in four cases. Only two cases possessed *HMGA2* fusion genes. The mean age of *LIFR–PLAG1*-positive cases was significantly higher than that of *CTNNB1–PLAG1* and *CHCHD7–PLAG1*-positive cases (*P* = 0.0358). PAs located in the submandibular gland demonstrated *CTNNB1–PLAG1* fusion at a significantly higher rate than other fusions (*P* = 0.0109). Histologically, *PLAG1* fusion-positive cases exhibited chondroid formation and plasmacytoid features more commonly (*P* = 0.043, *P* = 0.015, respectively) and myxoid abundant feature less frequently (*P* = 0.031) than *PLAG1* fusion-negative cases. For CXPAs, four *CTNNB1–PLAG1* fusions were detected in two salivary duct carcinomas and two myoepithelial carcinomas. Ductal formation was observed frequently (90.9%) in residual PA.

**Conclusions:** The presence of *PLAG1* fusion was associated with specific histological features in PA. Detecting the *PLAG1* fusion gene and searching residual ductal formation in salivary gland malignant tumours with extensive hyalinisation could be useful for diagnosis.

**Introduction**

Pleomorphic adenoma (PA) is the most frequent benign tumour of the salivary glands, accounting for 60% of all salivary gland tumours. Although it mainly arises in the parotid gland, the submandibular gland, buccal mucosa and minor salivary glands, including the soft palate, are also relatively common sites.\(^1\) PA occurs in individuals of all ages, and the mean age of patients with PA is 43 years.\(^2\)
Histologically, epithelial components such as ductal formation and squamous differentiation, and mesenchymal components such as myxoid stroma and chondroid matrix, are admixed within fine fibrous capsules. A modified myoepithelial origin of the two components was suggested, as a common clonality was found between the two components, and the proportion of those two elements varies widely among cases, demonstrating the morphological diversity of PAs. Some cases include only one predominant element; when this shows extreme cellularity and a minimal myxoid component, it is defined as ‘cellular PA’. However, the majority of PAs contain at least 30% myxoid components within the tumour; cellular PAs comprise only 12–15% of the total PAs.

Cytogenetic studies have shown that approximately 70% of PAs are karyotypically abnormal, with 25% of such abnormalities observed in 8q12, where the \textit{PLAG1} gene is located. Among \textit{PLAG1}-associated abnormalities, 40% are t(3;8)(p21;q12), resulting in the formation of a \textit{CTNNB1–PLAG1} gene fusion. Furthermore, \textit{CHCHD7}, \textit{LIFR} and \textit{TCEA1} have also been found as fusion partners of \textit{PLAG1}. The \textit{HMGA2} gene, also known as \textit{HMG1-C}, is well known as a gene involved in rearrangements of 12q14-15 in PAs, accounting for 8% of the tumours. So far, three fusion genes, \textit{HMGA2-NFIB}, \textit{HMGA2-FHIT} and \textit{HMGA2-WIF1} have been identified in PAs.

Although approximately half of PAs possess no chromosomal rearrangements involving the \textit{PLAG1} or \textit{HMGA2} genes, and \textit{LIFR–PLAG1} fusions have been reported in other tumours, \textit{PLAG1} and \textit{HMGA2} fusion genes appear to represent specific characteristics of PA. In PAs, \textit{PLAG1} protein levels are constantly elevated regardless of the \textit{PLAG1} fusion status. Therefore, the \textit{PLAG1} gene
is predicted to play an important role in the tumorigenesis of PA; however, how \textit{PLAG1} genes affect tumour morphology has not been fully examined.

Carcinoma ex pleomorphic adenoma (CXPA) was found to arise in PAs with a frequency of 6.2–9.5\% during a 20-year time-frame.\textsuperscript{1,17} Resection of PAs is the preferred treatment to prevent malignant transformation, as recurrence and persistence of the tumour are the two major risks.\textsuperscript{17} The most common histological subtype of CXPA is salivary duct carcinoma (SDC), followed by myoepithelial carcinoma (MYC) and adenocarcinoma not otherwise specified (NOS),\textsuperscript{1,18} although CXPA can present any histological subtype of salivary gland malignant tumour.\textsuperscript{1,19} Similar to PA, \textit{PLAG1}/\textit{HMGA2} rearrangements are the most frequently observed genetic events in CXPA,\textsuperscript{20} and detection of \textit{PLAG1} expression or \textit{PLAG1} rearrangements are practically used to distinguish PA and CXPA from other benign or malignant salivary tumours.\textsuperscript{20,21} \textit{PLAG1} is presumed to play an important role in CXPA; however, it is poorly understood, especially in the context of CXPA morphology.\textsuperscript{22–24}

In this study, we analysed \textit{PLAG1} and \textit{HMGA2} gene fusions in PAs by reverse transcription–polymerase chain reaction (RT–PCR) and examined the relationship between the fusion genes and the clinicopathological characteristics of PAs. Furthermore, we also examined these fusion genes in 11 cases of CXPA to investigate their relationship to the histopathology of malignant components and residual PA, but also investigated its influence on patients’ prognosis.

\textbf{Materials and methods}
SAMPLE PREPARATION AND CLINICAL INFORMATION

One hundred and five resected tumours of major (parotid, submandibular), minor (soft palate) salivary glands and lacrimal glands, diagnosed as pleomorphic adenoma (PA), and 11 resected tumours of major (parotid, submandibular) and minor (soft palate) salivary glands diagnosed as carcinoma ex PA (CXPA) were obtained at Juntendo University Hospital, Tokyo, Japan, between 2008 and 2013. Patients’ gender, age, tumour size (maximum diameter) and tumour location were obtained from clinical information. CXPAs were classified into non-invasive, minimally invasive and widely invasive, according to World Health Organisation (WHO) criteria.¹ We excluded CXPA samples in which residual PA components were only composed of hyalinized nodules lacking myoepithelial cells or epithelial/mesenchymal components to confirm the pathological diagnosis. Studies were performed according to the Declaration of Helsinki. This study was reviewed and approved by the Juntendo University School of Medicine Institutional Review Board (#2013069 and 2018055). This study is retrospective and non-invasive; therefore, individual informed written consent was not required.

RT–PCR

Four to five unstained slides 10 μm thick were prepared from formalin-fixed paraffin-embedded (FFPE) blocks for each case. After deparaffinising with xylene and ethanol, tumour tissue was separated by manual dissection and was put into 1.5-ml sterile microtubes. For homogenisation, 200 μl of lysis buffer (20 mM Tris-HCL, pH 8.0; 20 mM EDTA; and 2% sodium dodecyl sulphate) and 10 μl
of proteinase K (100 mg/ml) were added to each sample. After incubation at 55°C overnight, 1.0 ml of Trizol reagent (Gibco BRL, Gaithersburg, MD, USA) was added to the sample, and RNA was extracted as previously described. Five μl of total RNA was converted to cDNA by using the SuperScript first-strand synthesis system for RT–PCR (Invitrogen, Carlsbad, CA, USA), according to the manufacturers’ protocol. For PLAG1-associated fusion gene detection, a primer mixture of either CHCHD7, CTNNB1, LIFR or TCEA1 with PLAG1, and for HMGA2-associated fusion, a primer mixture of HMGA2 with either FHIT, NFIB or WIF1, was used. The primer sequences are described in the study by Matsuyama et al. PCR was performed using 9 μl of Platinum Blue PCR Supermix (Invitrogen) and 0.5 μl of 10 μmol/l of each primer. To check the quality of the cDNA, glyceraldehyde3-phosphate dehydrogenase (GAPDH) and beta-actin were used as internal controls. The PCR products were electrophoresed in 2.0% agarose gel and visualised with ethidium bromide. PCR products were cut from the gel and were sent for sequence analysis to Eurofins Genomics Inc. (Tokyo, Japan).

HISTOLOGICAL EVALUATION AND IMMUNOHISTOCHEMISTRY

Among 105 cases, there were 18 cases for which only one slide was available for pathological diagnosis, because of smaller tumour size (less than 2 cm). The other cases had two to nine slides/case. For these cases, we first checked all slides created from the maximum cut surface and selected one representative slide. This is based on finding that there was no apparent difference regarding the
histological components observed in each slide. However, when we found histological deviation between each slide, we also used other slides as references. Finally, one representative slide was chosen for each PA case and evaluated microscopically if any of the following histological components were present: ductal formation or squamous differentiation for epithelial components and chondroid formation or adipocytic differentiation for mesenchymal components (Figure 1A–D). Plasmacytoid differentiation was also evaluated (Figure 1E). Cases which included neither ductal formation nor squamous differentiation were counted as ‘no epithelial component’. PAs composed of tumour cells with high cellularity, where tumour cells occupied more than 80% of the mass and included no more than 10% of myxochondroid components, were characterised as ‘cellular PA’ (Figure 1L,M).\(^4,17\) In contrast, PAs rich in myxoid components, where myxoid components occupied at least 80% of the mass, were characterised as ‘myxoid abundant’ (Figure 1N). These two types of features were characterised as part of the histological evaluation of the tumours.

To evaluate the histopathological components, immunostaining of cytokeratin 7 (CK7), CK14 and S-100 was performed (Figure 1F–K). Internal ductal cells were stained by CK7, epithelial cells with squamous differentiation were stained by CK14 and chondroid cells in chondroid matrix were stained by S-100.\(^26,27\) Furthermore, we discriminated tumour cells with adipocytic differentiation from oval-like ducts by their negative staining for CK7. Plasmacytoid cells were stained by CK7 and S-100, as plasmacytoid cells were sometimes difficult to distinguish from plasma cells,\(^27\) and if there was any area of plasmacytoid differentiation it was counted as one.
The histopathological subtypes of malignant components of 11 CXPAs included salivary duct carcinomas (SDC) in six cases, myoepithelial carcinomas (MYC) in four cases and carcinosarcoma in one case. We estimated the percentage of residual PA and hyalinizing area in each case. The existence of histological components such as ductal formation, squamous differentiation, chondroid formation and adipocytic differentiation were evaluated by immunostaining in the same manner as for PAs, but were evaluated for each slide including those containing residual PA (Figure 2).

STATISTICAL ANALYSIS

The comparison of clinicopathological features between fusion-positive and -negative cases were examined by $\chi^2$ test or Fisher’s exact test and the Mann–Whitney $U$-test, as appropriate. The tumour-related survival time was measured from the date of diagnosis to the latest follow-up period or death due to salivary gland tumour. Survival curves were generated using the Kaplan–Meier method. $P$-values less than 0.05 were considered significant.

Results

CLINICAL FACTORS

The PA patient population in this study consisted of 40 males and 65 females, with ages ranging from 15 to 78 years (mean = 45.4, median ± range = 42 ± 14.9). Eighty-eight tumours were located in the parotid glands, 14 in the submandibular glands, two in the lacrimal glands and one in the minor glands.
of the soft palate. In two patients, samples were obtained from their first and fourth recurrence (both female, aged 33 and 74 years, respectively). The CXPA patient population consisted of five males and six females, ranging in age from 51 to 77 years (mean = 64.9, median ± range = 66 ± 8.16). Seven tumours were located in the parotid glands, four in the submandibular glands and one in the minor glands of the soft palate.

FUSION GENE ANALYSIS

The results of \textit{PLAG1} and \textit{HMGA2} fusion gene analyses are summarised in Table 1 (PA) and Table 2 (CXPA). Among 105 PAs, 40 cases (38.1\%) harboured \textit{PLAG1} fusion genes. Among 22 cases with a \textit{CTNNB1–PLAG1} fusion, 18 cases had \textit{CTNNB1} (exon 1)–\textit{PLAG1} (exon 2), and the remaining four cases had \textit{CTNNB1} (exon 1)–\textit{PLAG1} (exon 3). All cases with \textit{CHCHD7–PLAG1} fusions were \textit{CHCHD7} (exon 1)–\textit{PLAG1} (exon 2). \textit{LIFR–PLAG1} fusion transcripts were amplified in four cases; two cases were \textit{LIFR} (exon 1)–\textit{PLAG1} (exon 2) and two were \textit{LIFR} (exon 1)–\textit{PLAG1} (exon 3). Regarding \textit{HMGA2} fusion genes, two cases were positive for \textit{HMGA2} (exon 3)–\textit{WIF1} (exon 10). No fusion transcripts were amplified using the primer pairs for \textit{TCEA–PLAG1}, \textit{HMGA2–FHIT} or \textit{HMGA2–NFIB} fusion transcripts in any of the cases.

In CXPAs, four of 11 cases (36.4\%) – two SDCs and two MYC – harboured \textit{CTNNB1} (exon 1)–\textit{PLAG1} (exon 3). Other \textit{PLAG1} fusion variants and \textit{HMGA2} fusions were not detected.
CLINICAL RELATIONSHIPS WITH FUSION GENES

Only two cases were positive for HMGA2-related fusion genes. These cases arose in female patients aged 31 and 32 years. Both had tumours located in the parotid gland, which were 30 mm in maximum diameter. Because PLAG1 and HMAG2 anomalies exist in a mutually exclusive manner in PAs,28 we excluded these two HMGA2 fusion-positive cases from the clinicopathological analysis. We divided the remaining cases into PLAG1 fusion-positive and -negative groups and compared each group with a number of clinical factors. This analysis revealed no significant differences between two groups for gender, age and tumour location (Supporting information, Table S1). The tumour sizes located in parotid and submandibular glands did not correlate with PLAG1 fusion gene status (Supporting information, Table S2). Conversely, within PLAG1 fusion gene cases, the mean age of the LIFR–PLAG1 positive cases was significantly higher than that of cases involving the other two fusion partners (Table 3). Additionally, all PAs located in the submandibular gland harboured CTNNB1–PLAG1 fusion genes, and this difference was statistically significant ($P = 0.0109$). Gender and tumour size limited to parotid gland were also examined within each individual PLAG1 fusion gene partners, but no variable differences were found (Table 3 and Supporting information, Table S2). Among two recurrent cases, one case possessed the CTNNB1–PLAG1 fusion gene. There was no correlation between PLAG1 fusion and recurrence rate.

For CXPA, there were no significant differences between PLAG1 fusion-positive or -negative cases for age (fusion-positive cases: mean = 67.2, median ± range = 66 ± 4.71; fusion-negative cases:
HISTOPATHOLOGICAL RELATIONSHIPS WITH FUSION GENES

The frequencies of each histological component in PAs are described in Table 4. Correlations between histological features and \textit{PLAG1} fusion status are also described in Table 4 (two HMGA2 fusion-positive cases were excluded from this analysis). Overall, ductal formation, squamous differentiation and chondroid formation were observed in more than half the cases (91.4, 67.6 and 57.1%, respectively), although ‘no epithelial components’ cases and cellular PAs showed such features at a frequency of less than 5% (2.9 and 2.9%, respectively). Chondroid formation and plasmacytoid differentiation was observed more frequently in \textit{PLAG1} fusion-positive cases than in \textit{PLAG1} fusion-negative cases ($P = 0.043$, $P = 0.015$). In addition, ‘myxoid abundant’ features were observed significantly more frequently in \textit{PLAG1} fusion-negative cases than in \textit{PLAG1} fusion-positive cases ($P = 0.031$). Interestingly, among 34 ‘myxoid abundant’ cases, \textit{PLAG1} fusion-positive cases tended to show adipocytic features more often than PLAG1 fusion-negative cases (three of eight, 37.5 versus two of 26, 7.7%, respectively, $P = 0.0721$), although were not statistically significant. The presence of epithelial components did not correlate with the presence of a \textit{PLAG1} fusion gene.

The histopathological data for CXPA cases are shown in Table 2. Two minimally invasive and two widely invasive cases harboured a \textit{CTNNB1–PLAG1} fusion. There was no correlation between the
\textit{PLAG1} fusion status and histopathological subtypes. Ductal formation was most frequently observed (90.9\%) in residual PA and was present in all cases except case 5, in which extensive hyalinisation was evident. In addition, plasmacytoid differentiation was seen in approximately half the cases (54.5\%), followed by chondroid formation and squamous differentiation (45.5\% and 36.4\%, respectively). Adipocytic differentiation and ‘myxoid abundant’ were not observed, but cellular PA features were seen in one of the cases (case 9). Residual PAs in \textit{PLAG1} fusion-positive cases generally did not demonstrate plasmacytoid differentiation, although \textit{PLAG1} fusion-positive CXPAs tended to show chondroid formation (75\% of fusion-positive versus 28.6\% of fusion-negative cases).

The percentage of residual PA ranged from 1 to 30\% (average: 12.6\%). There was one case (case 5) with less than 1\%, in which a few benign myoepithelial cells were seen scattered in a widely hyalinizing stroma. The hyalinisation percentage ranged from 5 to 70\% (average: 37.7\%). Cases with extensive hyalinisation (60\% and more) and with minimal residual PA (not more than 10\%) tended to show fewer histological variations than the other cases (on average, 1.25 components versus 2.86).

\textbf{Discussion}

The genetic characteristics of PAs are well documented by means of spectral karyotype analysis,\textsuperscript{9} FISH\textsuperscript{16,20} or CISH.\textsuperscript{23} We compared our RT–PCR results with Matsuyama et al.’s data\textsuperscript{25} in order to evaluate the precise frequency of genetic alterations including cryptic fusions. The \textit{PLAG1} fusion-positive rate (38.1\%) in our study was higher compared to theirs (11 of 45 cases, 24.4\%). Specifically,
they found CTNNB1–PLAG1 fusions in eight of 45 cases (18%), CHCHD7–PLAG1 in one of 45 cases (2.2%) and LIFR–PLAG1 in two of 45 cases (4.4%). In contrast to our findings, they did not detect a TCEA–PLAG1 fusion in any of their cases. With the exception of CHCHD7–PLAG1, the frequencies of PLAG1 fusion genes in our study were comparable to theirs. Regarding the HMGA2 fusion genes, we identified two cases (two of 105, 1.9%) with HMGA2 (exon 3)–WIF1 (exon 10). Matsuyama’s group detected one case with HMGA2 (exon 3)–WIF1 (exon 9) (one of 45, 2.2%).

In addition to PA, representative tumour-specific fusion genes have also been reported in other salivary gland tumours, such as MYB–NFIB in adenoid cystic carcinoma, CRTCl–MAML2 in mucoepidermoid carcinoma, EWSR1–ATF1 in low-grade hyalinizing clear cell carcinoma and ETV6–NTRK3 in mammary analogue secretory carcinoma.\textsuperscript{29} CRTCl–MAML2 has been revealed once as a preferable prognostic factor among mucoepidermoid carcinoma, although recent studies identified it as a diagnostic rather than prognostic marker.\textsuperscript{30,45,46} We investigated the relationship of PLAG1 fusion status and the recurrence rate of PA, but no correlations were observed regarding the cancer-related survival time in CXPA. Recurrent fusion genes involving PLAG1 have been also described in soft tissue tumours such as lipoblastomas, in which COL1A2–PLAG1,\textsuperscript{31} HAS2–PLAG1,\textsuperscript{31} COL3A2–PLAG1\textsuperscript{32} and RAB2A–PLAG1\textsuperscript{32} have been identified. We examined these four PLAG1 fusions by RT–PCR, but no fusion transcripts were detected in any of our cases.

Previous studies have indicated that patients with karyotypically normal PAs are significantly older than those with rearrangements of 8q12.\textsuperscript{6} Our study did not yield similar data, although it is
interesting to note that patients with LIFR–PLAG1 fusions were older than those with fusions involving the other two PLAG1 partners. More interestingly, half the tumours (seven of 14 cases) in submandibular glands harboured PLAG1 fusions, and all of them were CTNNB1–PLAG1 fusions. Two cases from the lacrimal gland were included in our study, and both cases were positive for a PLAG1 fusion (CTNNB1–PLAG1 and CHCHD7–PLAG1). PA is the most common epithelial tumour of the lacrimal gland and constitutes more than 50% of primary tumours.33 Von Holstein34 demonstrated that epithelial tumours such as PA, adenoid cystic carcinoma, carcinoma ex PA (CXPA) and mucoepidermoid carcinoma of the lacrimal gland are molecularly very similar to their salivary gland counterparts in the expression of tumour-specific fusion genes and genomic imbalances demonstrated by array CGH. However, tumours of the lacrimal glands are extremely rare, and clinical or genetic studies involving a large number of cases are unavailable.

The impact of fusion genes on the histology of PAs has not previously been evaluated. PLAG1 fusion-positive cases tended to show frequent ‘chondroid formation’ and ‘plasmacytoid differentiation’ but less frequent ‘myxoid abundant’ features compared to PLAG1 fusion-negative cases. In line with this finding, Mark et al.6 described that PAs with normal karyotypes are often more stroma-rich than tumours with 8q12 abnormalities. In other tumours at other sites, clinicopathological data, including morphology, have been documented to correlate with some specific fusion genes. EML4–ALK in lung carcinoma is detected more in never-smoker patients of younger age with mucinous cribriform morphology.35,36 For soft tissue tumours, SS18–SSX1 is commonly detected in synovial sarcoma with
glandular epithelial differentiation, and in benign fibrous histiocytoma ALK rearrangement is restricted to epithelioid subtype. In Ewing sarcoma/undifferentiated round cell sarcoma, fusion partners to EWSR1 seem to be associated with patient age and location.

Matsuyama et al. examined the localisation of the PLAG1 protein by immunohistochemistry in 45 cases of PAs. According to their study, PLAG1 was found in mesenchymal components such as spindle cells, stellate cells, chondromatoid cells and plasmacytoid cells, but not in epithelial components such as glandular and squamoid cells. However, a recent study described that PLAG1 expression was observed in both epithelial and myoepithelial cells in PAs, with a higher prevalence in myoepithelial cells. In this study, epithelial components (ductal formation and squamous formation) were observed regardless of the PLAG1 fusion status; however, mesenchymal components such as chondroid formation and plasmacytoid differentiation varied according to the PLAG1 fusion status. In addition, given that PLAG1 fusion-positive ‘myxoid abundant’ PAs tended to show adipocytic differentiation, PLAG1 fusion-positive PAs demonstrated a more diverse morphology than the negative cases, although we have not performed PLAG1 immunohistochemistry. PAs with ‘no epithelial components’ were rare in both PLAG1 fusion-positive/-negative cases (5 and 1.6%) in this study. We confirmed the diagnosis of ‘no epithelial components’ PAs by searching for other features such as chondroid formation and adipocytic differentiation and excluded the possibility of myoepithelioma. Myoepithelioma is a rare benign salivary tumour with very close morphology to PA. During this study we found six myoepitheliomas diagnosed originally as PA, but revised as myoepitheliomas because of
lacing epithelial differentiation and other supporting evidence. We additionally performed RT–PCR for these six myoepitheliomas; two cases possessed CTNNB1–PLAG1 and one case CHCHD7–PLAG1. These cases included no ductal or chondroid components in any of the slides. This finding may raise the possibility of myoepithelioma being a tumour of the same entity as PA, in line with a study that one myoepithelioma was shown cytogenetically to have a 12q alteration. More cytological or genetic studies are needed to confirm this hypothesis.

Cellular PAs were extremely rare and seen at a similar frequency among PLAG1 fusion-positive and -negative groups (2.5 and 3.2% respectively), although only one of three cellular PAs showed PLAG1 rearrangements. Morphologically, cellular PAs bear a strong resemblance to basal cell adenomas (BCAs), thus this lower frequency of PLAG1 fusions in cellular PAs suggests that BCAs might have been misclassified as cellular PAs in previous studies. Recently, nuclear β-catenin expression has been reported as a highly specific and reliable marker for BCA. Therefore, we checked nuclear β-catenin expression in all cellular PA cases and confirmed negative staining.

We examined the fusion gene status in 11 cases of CXPA, and four of 11 cases (36.4%) harboured PLAG1 fusion genes. The frequency of PLAG1 fusions in CXPA was similar to that in PA (38.7%). Five cases of CXPA contained only a small amount of residual PA (≦ 5%), and among these cases, two (cases 4 and 8) contained CTNNB1–PLAG1. The detection of a PLAG1 fusion gene may indeed be a supplementary tool for diagnosis of CXPA. There were no significant differences in clinical factors including age, sex, tumour location and tumour size associated with the PLAG1 fusion status.
in CXPA. In PA, half the cases from the submandibular gland contained the \textit{CTNNB1–PLAG1} gene fusion; however, only one of three CXPA cases (33.3\%) from the submandibular gland harboured \textit{CTNNB1–PLAG1}. No fusion transcription was identified in CXPA for \textit{LIFR–PLAG1}, so the association between the patient’s age and the LIFR–PLAG1 fusion status could not be evaluated. There are two conflicting theories concerning PLAG1 expression in CXPA; one is that \textit{PLAG1} gene alterations in PAs are maintained even after malignant change\textsuperscript{11,24,25,44} and the other is that PLAG1 expression could be lost when PA undergoes malignant transformation.\textsuperscript{42} According to the latter theory, PLAG1 expression could be lost in carcinoma components of CXPA, especially in carcinoma with epithelial differentiation, but remain present mainly in low-grade carcinomas such as myoepithelial carcinoma and epithelial–myoepithelial carcinoma although, in that study, they defined abundant hyalinisation as residual PA components and included these cases as CXPA. Thus, the possibility of \textit{de-novo} salivary malignant tumours being included as CXPA could not be excluded. We found no correlation between \textit{PLAG1} fusion status and the histological subtype of CXPA; however, because of the small sample size it is too early to draw any conclusions.

There was difficulty in evaluating the histology of residual PA within CXPA, especially in cases with extensive hyalinisation. Extensive hyalinisation may have affected the evaluation of the residual PA components, as cases with extensive hyalinisation (60\% and more) not only included smaller areas of residual PA (not more than 10\%) but also showed fewer histological variations than others (1.25 versus 2.86). Ductal formation was the most frequently observed histological component
of residual PAs in CXPA (90.9%). Based on this finding, an auxiliary diagnostic tool for CXPA may be to find ‘ductal components without atypia’ in cases of salivary malignant tumours with extensive hyalinisation. The tendency to plasmacytoid differentiation observed in PLAG1 fusion-positive PAs was not evident in residual components of PLAG1 fusion-positive CXPAs, although chondroid formation was commonly observed in both PLAG1 fusion-positive cases (65.1% in PAs, 75% in residual PA of CXPAs). Myxoid components of residual PAs may have been invaded by malignant components and disappeared during tumour extension, and this may be one of the reasons for the absence of ‘myxoid abundant’ features in residual PAs. In line with this hypothesis, as plasmacytoid myoepithelial cells were frequently observed in nests or scattered in myxoid matrix, the disappearance of myxoid matrix by tumour extension might be associated with the decrease of plasmacytoid cells. Additionally, the absence of ‘myxoid abundant’ features in residual PAs is in line with the absence of adipocytic differentiation, as PLAG1 fusion-positive ‘myxoid abundant’ PAs tended to show adipocytic differentiation. There was only one case (case 9) of residual PA in which cellular PA features were observed (9.1%). This represents a fairly high rate compared to that in PAs (9.1 versus 2.7%), although this frequency may not reflect the true frequency due to the small sample size. From a genetic viewpoint, cellular PA may represent a genetic subgroup distinct from typical PA20 and, if so, it may develop different morphological features from CXPA when undergoing malignant changes. Further studies are needed to elucidate its characteristics.

In conclusion, several clinicopathological characteristics, including histological
differentiation, were related to \textit{PLAG1} fusions in PAs. \textit{PLAG1} fusion-positive PAs showed a more diverse morphology than the negative cases, suggesting that \textit{PLAG1} fusion is a determinant of morphological and histological features in PAs. Searching for ductal components could be a practical hallmark to diagnose CXPA in salivary gland malignant tumours with extensive hyalinisation.

\textbf{Acknowledgements}

The authors thank Michiko Takahashi and Ikuyo Yamamoto for their excellent technical assistance.

\textbf{Conflicts of interest}

The authors declare that there are no conflicts of interest.

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**Figure 1.** Pleomorphic adenoma (PA) showing the haematoxylin and eosin staining of each component of pleomorphic adenoma: **A,** ductal formation, **B,** squamous differentiation, **C,** chondroid formation, **D,** adipocytic differentiation, **S,** plasmacytoid differentiation. Immunohistochemistry used to determine each component. Cytokeratin 7 (CK7) (**F–H**), CK14 (**I**), S-100 (**J,K**). ‘Cellular PA’ (**L,M**) and ‘myxoid abundant’ PA (**N**). ‘Cellular PA’ is packed with tumour cells, and only scarce myxoid components can be seen. Conversely, ‘myxoid abundant’ PA consists mainly of chondro-myxoid components, and tumour cell islets are only observed in low numbers.

**Figure 2.** Microscopic views of carcinoma ex PA (CXPA), case eight. **A,** Myoepithelial carcinoma (MYC) components with necrosis is observed on the right, and myoepithelial cells without atypia in myxoid stroma are evident on the left, suggesting the presence of pleomorphic adenoma (PA). **B,** In this case, in a broad area of hyalinisation, there is a scarce area of PA containing ductal and chondromatoid components. These components were identified by immunohistochemistry as ductal components are positive for cytokeratin 7 (CK7) (**C**), and chondroid cells are positive for S-100 (**D**).
Table 1. Fusion gene types in this series of pleomorphic adenomas

<table>
<thead>
<tr>
<th>Fusion genes</th>
<th>Frequency</th>
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<tbody>
<tr>
<td>CTNNB1–PLAG1</td>
<td>22/105 (21%)</td>
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<tr>
<td>CHCHD7–PLAG1</td>
<td>14/105 (13.3%)</td>
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<tr>
<td>LIFR–PLAG1</td>
<td>4/105 (3.8%)</td>
</tr>
<tr>
<td>TCEA–PLAG1</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>40/105 (38.1%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fusion genes</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMGA2–WIF1</td>
<td>2/105 (1.9%)</td>
</tr>
<tr>
<td>HMGA2–FHIT</td>
<td>0</td>
</tr>
<tr>
<td>HMGA2–NFIB</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>2/105 (1.9%)</td>
</tr>
</tbody>
</table>

Table 2. Clinicopathological data of CXPA with PLAG1 fusion status and histology of residual PA

<table>
<thead>
<tr>
<th>No.</th>
<th>Age/sex</th>
<th>Location</th>
<th>Size (mm)</th>
<th>Category</th>
<th>Subtype</th>
<th>Fusion gene</th>
<th>Residual PA (%)</th>
<th>Hyalinisation (%)</th>
<th>Histological features of PA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>77/F</td>
<td>Parotid gland</td>
<td>35</td>
<td>Non-invasive (intracapsular)</td>
<td>NE</td>
<td>CTNNB1–PLAG1</td>
<td>10</td>
<td>60</td>
<td>d, p</td>
</tr>
<tr>
<td>2</td>
<td>66/M</td>
<td>Parotid gland</td>
<td>25</td>
<td>Minimally invasive</td>
<td>SDC</td>
<td>CTNNB1–PLAG1</td>
<td>20</td>
<td>30</td>
<td>d, c, p</td>
</tr>
<tr>
<td>3</td>
<td>57/M</td>
<td>Submandibular gland</td>
<td>25</td>
<td></td>
<td>NE</td>
<td>CTNNB1–PLAG1</td>
<td>30</td>
<td>5</td>
<td>d, p</td>
</tr>
<tr>
<td>4</td>
<td>74/F</td>
<td>Submandibular gland</td>
<td>28</td>
<td></td>
<td>MYC</td>
<td>CTNNB1–PLAG1</td>
<td>1</td>
<td>65</td>
<td>d, s</td>
</tr>
<tr>
<td>5</td>
<td>51/M</td>
<td>Parotid gland</td>
<td>38</td>
<td></td>
<td>NE</td>
<td>CTNNB1–PLAG1</td>
<td>10</td>
<td>30</td>
<td>d, p</td>
</tr>
<tr>
<td>6</td>
<td>56/M</td>
<td>Parotid gland</td>
<td>40</td>
<td></td>
<td>SDC</td>
<td>CTNNB1–PLAG1</td>
<td>&lt;1</td>
<td>70</td>
<td>*</td>
</tr>
<tr>
<td>7</td>
<td>66/M</td>
<td>Parotid gland</td>
<td>42</td>
<td>Widely invasive</td>
<td>MYC</td>
<td>CTNNB1–PLAG1</td>
<td>20</td>
<td>25</td>
<td>d, s, c, p</td>
</tr>
<tr>
<td>8</td>
<td>63/F</td>
<td>Parotid gland</td>
<td>45</td>
<td></td>
<td>MYC</td>
<td>CTNNB1–PLAG1</td>
<td>5</td>
<td>45</td>
<td>d, s, c</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>66/F</td>
<td>Parotid gland</td>
<td>40</td>
<td>NE</td>
<td>20</td>
<td>20</td>
<td>d, s, p, ce</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>77/F</td>
<td>Minor salivary gland</td>
<td>40</td>
<td>NE</td>
<td>5</td>
<td>5</td>
<td>d, c, p</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>61/F</td>
<td>Submandibular gland</td>
<td>30</td>
<td>Carcinosarcoma</td>
<td>NE</td>
<td>5</td>
<td>60</td>
<td>d, c</td>
<td></td>
</tr>
</tbody>
</table>

SDC, salivary duct carcinoma; MYC, myoepithelial carcinoma; NE, negative; d, ductal formation; s, squamous differentiation; c, chondromatoid formation; p, plasmacytoid differentiation; ce, cellular pleomorphic adenomas (PA); *few myoepithelial cells were observed; NED: no evidence of disease; AWD: alive with disease; DOD: died of disease; CXPA, carcinoma ex PA.
TNM, tumour–node–metastasis; NED, no evidence of disease; DOD, died of disease; AWD, alive with disease.

**Table 3.** Correlation between PLAG1 fusion partner and clinical factors

<table>
<thead>
<tr>
<th>PLAG1 fusion partner (n = 40)</th>
<th>CTNNB1 (n = 22)</th>
<th>CHCHD7 (n = 14)</th>
<th>LIFR (n = 4)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male (n = 12)</td>
<td>8 (66.7%)</td>
<td>4 (33.3%)</td>
<td>0</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Female (n = 28)</td>
<td>14 (50%)</td>
<td>10 (35.7%)</td>
<td>4 (14.3%)</td>
<td></td>
</tr>
<tr>
<td>Age*</td>
<td>44.1/42.5 ± 13.5</td>
<td>42.9/42.0 ± 15.7</td>
<td>61.0/63.0 ± 12.2</td>
<td>0.0358**</td>
</tr>
<tr>
<td>(23-72)</td>
<td>(15-64)</td>
<td>(43-75)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parotid (n = 31)</td>
<td>14 (45.2%)</td>
<td>13 (41.9%)</td>
<td>4 (12.9%)</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Submandibular (n = 7)</td>
<td>7 (100%)</td>
<td>0</td>
<td>0</td>
<td>0.0109***</td>
</tr>
</tbody>
</table>

TNM, tumour–node–metastasis; NED, no evidence of disease; DOD, died of disease; AWD, alive with disease.
<table>
<thead>
<tr>
<th>Histological features</th>
<th>All cases ((n = 105))</th>
<th>PLAG1 fusion gene* Positive ((n = 40))</th>
<th>Negative ((n = 63))</th>
<th>(P)-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ductal formation</td>
<td>96/105 (91.4%)</td>
<td>37 (92.5%)</td>
<td>57 (90.5%)</td>
<td>&gt; 0.99</td>
</tr>
<tr>
<td>Squamous differentiation</td>
<td>71/105 (67.6%)</td>
<td>27 (67.5%)</td>
<td>43 (68.3%)</td>
<td>&gt; 0.99</td>
</tr>
<tr>
<td>No epithelial components</td>
<td>3/105 (2.9%)</td>
<td>2 (5%)</td>
<td>1 (1.6%)</td>
<td>0.558</td>
</tr>
<tr>
<td>Chondroid formation</td>
<td>60/105 (57.1%)</td>
<td>28 (70%)</td>
<td>31 (49.2%)</td>
<td>0.043</td>
</tr>
<tr>
<td>Adipocytic differentiation</td>
<td>21/105 (20%)</td>
<td>11 (27.5%)</td>
<td>9 (14.3%)</td>
<td>0.126</td>
</tr>
<tr>
<td>Plasmacytoid differentiation</td>
<td>48/105 (45.7%)</td>
<td>24 (60%)</td>
<td>22 (34.9%)</td>
<td>0.015</td>
</tr>
<tr>
<td>Cellular</td>
<td>3/105 (2.9%)</td>
<td>1 (2.5%)</td>
<td>2 (3.2%)</td>
<td>&gt; 0.99</td>
</tr>
<tr>
<td>Myxoid abundant</td>
<td>34/105 (32.4%)</td>
<td>8 (20%)</td>
<td>26 (41.3%)</td>
<td>0.031</td>
</tr>
</tbody>
</table>

* Two cases with \(HMGA2\) fusions were excluded from this analysis.