Expression of p16\(^{\text{Ink4a}}\) protein in pleomorphic adenoma and carcinoma ex pleomorphic adenoma proves diversity of tumour biology and predicts clinical course

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ABSTRACT

Aims The aim of the study is to correlate p16\(^{\text{Ink4a}}\) expression with the clinical courses of pleomorphic adenoma (PA), its malignant transformation (CaexPA) and treatment outcomes.

Methods Retrospective analysis (1998–2019) of 47 CaexPA, 148 PA and 22 normal salivary gland samples was performed. PAs were divided into two subsets: clinically ‘slow’ tumours characterised by stable size or slow growth; and ‘fast’ tumours with rapid growth rate.

Results Positive p16\(^{\text{Ink4a}}\) expression was found in 68 PA and 23 CaexPA, and borderline expression in 80 and 20, respectively. All 22 (100%) normal salivary gland samples presented with no p16\(^{\text{Ink4a}}\) expression. Significant difference in p16\(^{\text{Ink4a}}\) expression was observed between normal tissue, PA and CaexPA (\(\chi^2\) (4)=172,19; \(p=0.0001\)). The PA clinical subgroups were also evaluated separately, revealing additional statistical relations: ‘fast’ PA and CaexPA differed significantly in p16\(^{\text{Ink4a}}\) expression (\(\chi^2\) (2)=8.06; \(p=0.0178\)) while ‘slow’ PA and CaexPA did not (\(\chi^2\) (2)=3.09; \(p=0.2129\)). 3-year, 5-year and 10-year survival among p16\(^{\text{Ink4a}}\) positive CaexPA patients was 100%, 90.56% and 60.37%, respectively, and in CaexPA patients with borderline p16\(^{\text{Ink4a}}\) expression was 90.0%, 73.64% and 22.20%, respectively. Statistically significant difference between expression pattern and survival rate was observed (F Cox test — F (16, 24)=2.31; \(p=0.03075\)).

Conclusions Our study confirms no p16\(^{\text{Ink4a}}\) expression in normal tissue, but reveals differences in expression between ‘fast’ and ‘slow’ PA. We suggest that p16\(^{\text{Ink4a}}\) overexpression is connected to PA proliferation and subsequent malignant transformation to CaexPA. Borderline p16\(^{\text{Ink4a}}\) staining correlates with worse prognosis of CaexPA.

INTRODUCTION

Pleomorphic adenoma (PA) is the most common salivary gland neoplasm, accounting for approximately 70% of tumours.\(^1\) While PA is a benign lesion, its diverse clinical course, recurrences, and risk of malignant transformation comprise a medical challenge.\(^2\) PAs are usually well circumscribed and encapsulated, often with tongue-like protrusions or occasional satellite nodules.\(^3\) Morphological patterns vary with three components: epithelial, mesenchymal and mixed.\(^4\) Foci of squamous cells are an integral feature of PA; however, extensive squamous metaplasia is uncommon and can be easily misinterpreted as squamous cell carcinoma.\(^7\)

In this paper, we present a new insight into a single histological unit: PA. Our 20-year experience of 1500 PAs and extensive observation of their individually variable disease courses has prompted us to distinguish two clinically divergent subsets: ‘fast’ and ‘slow’ tumours.\(^1\) While ‘fast’ PAs are characterised by a short medical history and rapid growth, ‘slow’ PAs demonstrate very stable biology and long-term growth. Progression, recurrence and malignant transformation are well-established PA behaviours, but the extremely fast growth of this benign tumour has always been a cause of concern for clinicians. Our team proved that the fast clinical course of PA has a great impact on further medical aspects.\(^7\) Thus, we undertake to search for immunohistochemical marker alterations among this single, clinically divergent histological unit.

Carcinoma ex PA (CaexPAs) arise from either primary or recurrent PAs, comprising 11.6% of all salivary gland malignancies,\(^1\) \(^2\) with a prevalence rate of 5.6 per 100 000.\(^9\) The clinical history is usually repetitive. After a long asymptomatic period, PAs start to grow rapidly and complaints such as pain, facial nerve palsy, and skin involvement may present.\(^2\) Longevity and recurrence seem to increase the risk of malignant transformation.\(^9\) This rate increases from 1.6% in tumours of <5 years, to 9.6% for tumours of >15 years.\(^11\) According to the latest WHO classification, CaexPA is no longer considered a stand-alone diagnosis.\(^13\) A substantial proportion of CaexPAs are now categorised as salivary duct carcinomas and myoepithelial CaexPAs.\(^14\)

Little is known about the genetic background and markers that characterise the PA-CaexPA malignant transformation, and the scant available data are inconsistent.\(^12\)\(^13\)\(^14\) Because malignant transformation to CaexPA is mostly derived from the epithelial component of PA,\(^17\) it is rational to investigate p16\(^{\text{Ink4a}}\) expression rather than other tumour suppressor proteins as their role in the development of head and neck squamous cell carcinoma (HNSCC) is proven. p16\(^{\text{Ink4a}}\) is a tumour suppressor protein that is substantially downregulated in many malignancies.\(^18\) Almost 50% of human carcinomas demonstrate loss of p16\(^{\text{Ink4a}}\), for example, non-human papillomavirus (HPV)-related
head and neck cancers, as well as pancreas, oesophagus, biliary tract, lung, liver, bladder, colon and breast carcinomas. On the other hand, p16\textsuperscript{INK4a} is also known to be overexpressed, namely in high-risk HPV-positive oropharyngeal and urogenital carcinomas.\textsuperscript{19–21} Oropharyngeal cancers (OPCs) comprise a subset of HNSCCs that arise from the oral cavity, oropharynx, hypopharynx, larynx and sinonasal tract and are anatomically limited to the base of the tongue, tonsils, posterior pharyngeal wall and soft palate.\textsuperscript{22} OPCs display two variant etiologies: tobacco and alcohol consumption for p16\textsuperscript{INK4a}-negative cancers, and high-risk HPV infection for p16\textsuperscript{INK4a}-positive cases.\textsuperscript{21} p16\textsuperscript{INK4a} investigation has become a practical alternative to oropharyngeal and urogenital HPV testing.\textsuperscript{22}

The PA neoplastic transformation remains ambiguous, and its progression to its malignant counterpart derives from the epithelial cells of PA. Thus we decided to investigate p16\textsuperscript{INK4a} immunohistochemical expression in PA and CaexPA. One of the tasks we undertook was to search within the PA group for molecular alterations that may reflect the observed differences in PA proliferation rate. Second, we investigated whether the level of p16\textsuperscript{INK4a} immunohistochemical protein expression in CaexPA could constitute a prognostic factor of the outcome.

Thus, the main goal of our study is to examine p16\textsuperscript{INK4a} immunohistochemical protein expression as a biomarker which may have an impact on the rate of proliferation of PA and CaexPA, and their respective clinical courses.

MATERIALS AND METHODS

Multicentre retrospective analyses of 47 CaexPA from four university hospitals in Poland were performed. Formalin-fixed paraffin-embedded (FFPE) blocks and available clinical data were collected from archives dating from 1998 to 2019. The second examined group consisted of 148 parotid PA cases (FFPE blocks and clinical data). The reference group consisted of normal salivary gland tissue (NSGT) (22 FFPE blocks).

Patients diagnosed with CaexPA did not undergo surgery prior to malignant transformation, thus the PA material from these patients was unobtainable. In summary, analyses were performed on 47 samples of CaexPA, 148 of PA and 22 of NSGT.

All patients provided written informed consent for participation. Every patient participated at each stage.

All histopathological examinations were performed by two experienced pathologists. Tissue microarray paraffin blocks were cut on a manual rotary microtome (AccuCut, Sakura, Torrance, USA) into 4 µm thick paraffin sections, and placed on extra-adhesive slides (SuperFrostPlus, MenzelGlas, Braunschweig, Germany). Immunohistochemistry (IHC) was standardised using a series of positive and negative control (HPV-positive SCC) reactions on FFPE tissue sections.

Immunohistochemical staining was performed using automated slide-processing system Benchmark GX Platform (Ventana Medical Systems, Tuscon, Arizona, USA) with primary mouse monoclonal antibody CINtec p16\textsuperscript{INK4a} antibody (clone E6H4, cat. no 705–4713; Ventana Medical Systems), and visualisation system UltraView DAB IHC Detection Kit (Ventana Medical Systems) in the procedure recommended by the manufacturer. Finally, the slides were dehydrated, cleared in a series of xylene, and coverslipped with Tissu-Tek (Sakura, Japan).

The pathologists independently evaluated the immunohistochemical expression of the examined antigens and were blinded to clinical and other data. In accordance with findings by Jordan et al, Bussu et al, and Cerezo et al, detailed in a systematic review by Frigge et al, we have scored the intensity of strong and diffuse nuclear and cytoplasmic staining of the p16\textsuperscript{INK4a} protein on a three-stage scale of p16\textsuperscript{INK4a} protein expression: 0—negative (no p16\textsuperscript{INK4a} expression) (figure 1), 1—borderline expression (1–69% of p16\textsuperscript{INK4a}-positive cells) (figure 2) and 2—positive expression (≥70% of p16\textsuperscript{INK4a}-positive cells) (figure 3). This division provided a comprehensive assessment of protein expression and a clearer understanding of the role of potential tumour markers in predicting outcome.\textsuperscript{15–16} Beside positive p16\textsuperscript{INK4a} expression, characteristic for high-risk HPV infection, we also implemented borderline expression of p16\textsuperscript{INK4a}, following the publications presenting no evidence of HPV infection in the aetiology of salivary gland neoplasms.\textsuperscript{24–29}

The primary outcome measure was the evaluation of p16\textsuperscript{INK4a} expression in tumour tissue, with special regard to CaexPA and the PA group divided into two subsets of ‘fast’ and ‘slow’ tumours. The following outcome measures was p16\textsuperscript{INK4a} expression with regard to other variables: age, gender, time of complaints, recent acceleration in tumour growth, type of symptoms, recurrence, observation time, distant metastases and death. The final outcome measure was the correlation between p16\textsuperscript{INK4a} expression and survival of CaexPA.

Figure 1 Expression of p16\textsuperscript{INK4a} protein in normal salivary gland—negative expression; nuclei counterstained with haematoxylin.

Figure 2 Expression of p16\textsuperscript{INK4a} protein in pleomorphic adenoma—positive expression; nuclei counterstained with haematoxylin.
Statistical analysis was performed using Statistica V.13. Descriptive statistics such as mean, minimum, maximum and SD were calculated for continuous variables. The χ² test was used for categorical data. Student’s t-test and correlation coefficient were used for continuous data. The level of significance was set at p<0.05. For multiple comparisons, the Bonferoni correction was used on the level p<0.0167.

RESULTS

There were 91 patients with positive p16Ink4a expression and 100 with borderline expression. Positive p16Ink4a expression was found in 68 PAs and 23 CaexPAs, and borderline expression in 80 and 20 cases, respectively. None of the 22 (100%) control cases of NSGT presented with p16Ink4a expression. No expression of p16Ink4a was found in the four remaining CaexPA patients (table 1).

Table 1 Percentage distribution of p16Ink4a immunohistochemical staining in tissue material: NSGT, PA (including division into ‘slow’ and ‘fast’ subsets) and CaexPA

| p16Ink4a expression | Normal | All PA | ‘Slow’ PA | ‘Fast’ PA | CaexPA | All PA and CaexPA |
|---------------------|--------|--------|-----------|-----------|--------|-------------------|
| Positive            | 0 (0.00%) | 68 (74.73%) | 32 (47.06%) | 36 (52.94%) | 23 (25.27%) | 91                 |
| Borderline          | 0 (0.00%) | 80 (80.00%) | 20 (25.00%) | 60 (75.00%) | 20 (20.00%) | 100                |
| No expression       | 22 (84.62%) | 0 (0.00%) | 0 (0.00%) | 0 (0.00%) | 4 (15.38%) | 26                 |
| Total               | 22 | 148 | 52 | 96 | 47 | 217 |

Carcinoma ex PA group

Of 47 CaexPA cases, there were 25 men (53.19%) and 22 women (46.81%). Mean age of the patients was 55.32±11.59 SD years, range 31–81 years. Mean size of the tumour was 44.11±24.90 SD mm, range 9–160 mm. Mean duration of symptoms was 110.57±112.90 SD months, range 60–360 months. Mean time of patient observation was 69 months. All CaexPA cases had occurred in the parotid gland (47 patients, 100%). Thirty patients (63.83%) underwent extended surgery, and 17 (36.17%) underwent surgery restricted to the salivary gland. Thirty-nine patients (82.98%) received adjuvant treatment while 8 (17.02%) did not.

Additionally, we analysed p16Ink4a expression in the course from NSGT to CaexPA via ‘slow’ PA, as well as from NSGT to CaexPA via ‘fast’ PA. Both ‘slow’ and ‘fast’ PA compared with unchanged tissue demonstrated a significant difference in p16Ink4a expression (p=0.00001). Analysis of p16Ink4a expression between ‘slow’ PA and CaexPA showed no differences (χ² (2)=3.09; p=0.2129), while a significant difference


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Figure 3 Expression of p16Ink4a protein in carcinoma ex pleomorphic adenoma—positive expression; nuclei counterstained with haematoxylin.
Original research

Table 2  Statistical analysis of p16\textsuperscript{ink4a} protein expression in reference to clinical data in the PA group

| p16\textsuperscript{ink4a} expression in PA | Positive | Borderline | Statistic | P value* |
|-----------------------------------------|----------|------------|-----------|---------|
| Age, years (mean 44.93) | 45.03 | 44.85 | t (146)=−0.07 | 0.9371* |
| Gender | | | | |
| Men (n=46) | 20 (43.48%) | 26 (56.52%) | χ\textsuperscript{2} (1)=0.16 | 0.6858b |
| Women (n=102) | 48 (47.06%) | 54 (52.94%) | | |
| Tumour size, mm (mean 30.05) | 29.65 | 30.40 | t (146)=0.26 | 0.7982* |
| Clinical course | | | | |
| ‘Fast’ (n=96) | 36 (37.50%) | 60 (62.50%) | χ\textsuperscript{2} (1)=7.84 | 0.0051b |
| ‘Slow’ (n=52) | 32 (61.54%) | 20 (38.46%) | | |
| Duration of symptoms, months (mean 48.62) | 59.24 | 39.60 | t (146)=−2.00 | 0.0478a |
| Recent acceleration | | | | |
| No (n=114) | 54 (47.37%) | 60 (52.63%) | χ\textsuperscript{2} (1)=0.40 | 0.5249b |
| Yes (n=34) | 14 (41.18%) | 20 (58.82%) | | |

Values have been bolded to highlight statistical significance.

*(a—Student’s t-test, b—χ\textsuperscript{2} test).
PA, pleomorphic adenoma.

Table 3  Statistical analysis of p16\textsuperscript{ink4a} protein expression in reference to clinical data in the CaexPA group

| p16\textsuperscript{ink4a} expression in CaexPA | Positive | Borderline | No expression | Statistic | P value* |
|-----------------------------------------------|----------|------------|---------------|-----------|---------|
| Age, years (mean 55.32) | 52.61 | 58.75 | 53.75 | H (2, N=47)=3.27 | 0.1946c |
| Gender | | | | | |
| Men (n=25) | 11 (44%) | 12 (48%) | 2 (8%) | χ\textsuperscript{2}(2)=2.17 | 0.33773b |
| Women (n=22) | 12 (54.5%) | 8 (36.4%) | 2 (9.1%) | | |
| Tumour size, mm (mean 44.11) | 43.39 | 46.25 | 37.5 | H (2, N=47)=1.40 | 0.4956c |
| Duration of symptoms, months (mean=110.57) | 95.87 | 119.60 | 150.0 | H (2, N=47)=0.20 | 0.9064c |
| Type of symptoms | | | | | |
| Malignant (n=24) | 13 (54.2%) | 8 (33.3%) | 3 (12.5%) | χ\textsuperscript{2}(2)=1.22 | 0.54355b |
| Benign (n=23) | 10 (34.4%) | 12 (52.2%) | 1 (4.4%) | | |
| Recurrence | | | | | |
| No (n=31) | 17 (54.8%) | 11 (35.5%) | 3 (9.7%) | χ\textsuperscript{2}(2)=2.17 | 0.33773b |
| Yes (n=15) | 6 (40%) | 8 (53.3%) | 1 (6.7%) | | |
| Distant metastases | | | | | |
| No (n=20) | 11 (55.0%) | 7 (35.0%) | 2 (10.0%) | χ\textsuperscript{2}(2)=1.71 | 0.17930b |
| Yes (n=20) | 8 (38.1%) | 12 (57.1%) | 0 (0.0%) | | |

*(b—χ\textsuperscript{2} test, c—Kruskal-Wallis test).
CaexPA, carcinoma ex pleomorphic adenoma.

between ‘fast’ PA and CaexPA was demonstrated (χ\textsuperscript{2} (2)=8.06; p=0.01781).

From these results, we conclude that the increased expression of p16\textsuperscript{ink4a} correlates with PA development, as well as with the malignant transformation from PA to CaexPA. The difference in p16\textsuperscript{ink4a} expression in the PA-to-CaexPA neoplastic pathway correlates with the clinical course of the benign PA precursor.

PA analysis
There was positive p16\textsuperscript{ink4a} expression in 68 (45.95%) cases and borderline p16\textsuperscript{ink4a} expression in 80 (54.05%).

We proved a significant difference in p16\textsuperscript{ink4a} expression in PA of variable clinical course (χ\textsuperscript{2} (1)=7.84; p=0.0051). Of the patients with positive p16\textsuperscript{ink4a} expression, slow growth of the tumour was reported in 32 (47.06%) cases, while fast growth was reported in 36 (52.94%). In patients with borderline expression, slow growth of the tumour was reported in 20 (25.00%), while fast growth was reported in 60 (75.00%). Borderline p16\textsuperscript{ink4a} expression in PAs is correlated with fast growth pattern. There was a significant difference in duration of symptoms between patients with positive and borderline p16\textsuperscript{ink4a} expression (Student’s t-test (146)=−2.00; p=0.0478). Mean duration of symptoms in patients with positive and borderline p16\textsuperscript{ink4a} expression was 59.24 and 39.60 months, respectively.

There was no significant difference between patients with positive and borderline expression of p16\textsuperscript{ink4a} in any other variables such as: age, gender, tumour size and recent growth acceleration (table 2).

CaexPA analysis
Of the 47 patients with CaexPA, 24 (51.06%) presented with a typical malignant clinical course (facial nerve palsy, pain, skin redness or ulceration), while 23 (48.94%) reported a lump that imitated a benign lesion (asymptomatic swelling only). Positive expression of p16\textsuperscript{ink4a} was demonstrated in 23 (48.94%), borderline expression in 20 (42.55%) and no expression in 4 (8.51%) patients. There was no significant difference in p16\textsuperscript{ink4a} expression with regard to any of following variables: age, gender, tumour size, duration of symptoms, type of symptoms, recurrence and distant metastases (table 3).
CaexPA survival analysis

Fifteen (31.91%) patients developed recurrence, and distant metastases were observed in 20 patients (42.55%). Twenty-one patients died (44.68%) and 26 (55.32%) were living at the end of follow-up.

Three-year survival was 93.48%, 5-year survival was 74.76% and 10-year survival was 44.01% for the whole CaexPA group. In patients with positive p16\textsuperscript{ink4a} expression survival was 100%, 90.56% and 60.37%, respectively; in patients with borderline p16\textsuperscript{ink4a} expression, 90.0%, 73.64% and 22.20%, respectively; and in patients with no p16\textsuperscript{ink4a} expression, 100%, 75% and 0%, respectively. A significant difference in CaexPA survival was observed when patients with no p16\textsuperscript{ink4a} expression, 100%, 75% and 0%, respectively; in patients with borderline p16\textsuperscript{ink4a} expression, 90.0%, 73.64% and 22.20%, respectively; and in patients with no p16\textsuperscript{ink4a} expression, 100%, 75% and 0%, respectively. A significant difference in CaexPA survival was observed when patients with no p16\textsuperscript{ink4a} expression, 100%, 75% and 0%, respectively; in patients with borderline p16\textsuperscript{ink4a} expression, 90.0%, 73.64% and 22.20%, respectively; and in patients with no p16\textsuperscript{ink4a} expression, 100%, 75% and 0%, respectively. A significant difference in CaexPA survival was observed when patients with no p16\textsuperscript{ink4a} expression, 100%, 75% and 0%, respectively; in patients with borderline p16\textsuperscript{ink4a} expression, 90.0%, 73.64% and 22.20%, respectively; and in patients with no p16\textsuperscript{ink4a} expression, 100%, 75% and 0%, respectively.

DISCUSSION

Our main results are concerned with p16\textsuperscript{ink4a} expression in NSGT, PA and CaexPA. There are no studies in the available literature comparing p16\textsuperscript{ink4a} expression with the clinical data of PAs and CaexPAs, or the oncological outcome of patients with CaexPA. Thus we outline the following study aims: to identify PAs and CaexPAs, or the oncological outcome of patients with worse 3, 5 and 10 years survival. Based on these results, we conclude that borderline p16\textsuperscript{ink4a} expression is related to bad prognosis (table 4, figure 4).

Table 4 The percentage distribution of p16\textsuperscript{ink4a} immunohistochemical staining in the CaexPA group in reference to survival rate

| p16\textsuperscript{ink4a} expression in CaexPA | Positive, % | Borderline, % | No expression, % |
|---------------------------------------------|-------------|---------------|-----------------|
| Survival, years                             |             |               |                 |
| 3                                           | 90.54       | 73.64         | 75              |
| 5                                           | 90.37       | 60.37         | 22.20           |
| 10                                          | 60.37       | 32.20         | 0.0             |

CaexPA, carcinoma ex pleomorphic adenoma.

Three- year survival was 93.48%, 5- year survival was 74.76% and 10- year survival was 44.01% for the whole CaexPA group. In patients with positive p16\textsuperscript{ink4a} expression survival was 100%, 90.56% and 60.37%, respectively; in patients with borderline p16\textsuperscript{ink4a} expression, 90.0%, 73.64% and 22.20%, respectively; and in patients with no p16\textsuperscript{ink4a} expression, 100%, 75% and 0%, respectively. A significant difference in CaexPA survival was observed when patients with no p16\textsuperscript{ink4a} expression, 100%, 75% and 0%, respectively; in patients with borderline p16\textsuperscript{ink4a} expression, 90.0%, 73.64% and 22.20%, respectively; and in patients with no p16\textsuperscript{ink4a} expression, 100%, 75% and 0%, respectively.

Figure 4 Kaplan- Meier probability of survival correlating p16\textsuperscript{ink4a} expression and CaexPA survival. CaexPA, carcinoma ex pleomorphic adenoma.
gland lumps, and also show a significant difference in p16\(^{ink4a}\) expression between NSGT and both benign and malignant salivary gland tumours.

An innovative approach in our work was to divide benign PAs into ‘fast’ and ‘slow’ categories according to clinical features. Our study has confirmed molecular differences between tumours that appear to be histologically identical. Borderline p16\(^{ink4a}\) expression correlates with ‘fast’ PA progression. Most importantly, the difference between CaexPA and ‘fast’ PA, when considered separately from PA as a whole, was statistically significant, while ‘slow’ PA was not. These results indicate that the difference in p16\(^{ink4a}\) expression in the neoplastic pathway between PA and CaexPA correlates with the clinical course of the benign PA precursor.

To summarise, we have successfully implemented the hypothesis of our work comparing the gradual stages of cancer transformation from NSGT, via clinically ‘slow’ and ‘fast’ subsets of PA, to CaexPA. Our results reveal that NSGT did not express p16\(^{ink4a}\) and that the p16\(^{ink4a}\) expression level in PA correlates with slow or fast PA clinical behaviour. Moreover, the p16\(^{ink4a}\) expression level was revealed to hold prognostic value for patients with CaexPA. Borderline p16\(^{ink4a}\) expression is related to worse survival.

**Take home messages**

- p16\(^{ink4a}\) expression gradually increases in the neoplastic pathway from normal salivary gland tissue via pleomorphic adenoma (PA) to carcinoma ex PAs (CaexPA).
- p16\(^{ink4a}\) overexpression correlates with the proliferation of PA and subsequent malignant transformation to CaexPA.
- There is a statistically significant difference in p16\(^{ink4a}\) expression among PA with variable clinical courses.
- The level of p16\(^{ink4a}\) expression constitutes a prognostic value for patients with CaexPA.

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