The Expression of FHIT in Salivary Carcinoma Ex Pleomorphic Adenoma

OMAR KUJAN[1,3], BASSEL TARAKJI[2,4], NALIN THAKKER[5,6], ABDUL AZIZ AL KHERAIF[7] and PHILIP SLOAN[8]

Departments of [1]Biomedical Sciences, and [2]Oral Diagnostic Sciences, School of Dentistry, AlFarabi College, Riyadh, Saudi Arabia; [3]Department of Oral Pathology, School of Dentistry, Al-Baath University, Hama, Syria; [4]Department of Oral Pathology, School of Dentistry, Aleppo University, Aleppo, Syria; [5]Department of Genetic Medicine, School of Medicine and [6]School of Dentistry, University of Manchester, Manchester, U.K.; [7]Dental Biomaterials Research Chair, Dental Health Department, College of Applied Medical Sciences, King Saud University, Riyadh, Saudi Arabia; [8]School of Dental Sciences, University of Newcastle, Newcastle upon Tyne, U.K.

Abstract. *Background: Carcinoma ex-pleomorphic adenoma (Ca-ex-PA) is considered to be a malignant transformation product of pre-existing pleomorphic salivary adenoma (PSA). Aim: Our study aimed to characterise alterations in the immunohistochemical expression of the Fragile Histidine Traid (FHIT) and Cyclin-dependent Kinase Inhibitor 2A (CDKN2A) ($p16^{INK4a}$) genes during tumour progression model from PSA to Ca-ex-PA in a cross sectional study. Materials and Methods: Paraffin blocks of 29 cases of PSA which were surrounded by normal parotid gland, and 26 cases of Ca-ex-PA were retrieved and validated. In all cases of Ca-ex-PA, a PSA 'ghost' was identified and the malignant element was either undifferentiated carcinoma or adenocarcinoma. Immunohistochemical staining and evaluation for CDKN2A and FHIT in 55 specimens were undertaken. Results: The results showed positive nuclear expression of p16 and FHIT in normal parotid gland. None (0%) of the PSA cases demonstrated loss of expression of nuclear FHIT, while 6/26 (23.1%) showed loss of FHIT express. Loss of CDKN2A expression was found in 12/29 (41.4%) of PSAs and 8/26 (30.8%) of Ca-ex-PAs. The nuclear expression pattern for FHIT was significantly more frequent in Ca-ex-PAs compared to PSAs (p=0.014). Conclusion: Our data suggest that inactivation of tumour suppressor genes plays an important role in the evolution of Ca-ex-PA. Furthermore, alteration of CDKN2A expression was found to be an early event in the malignant transformation of pleomorphic adenoma and could be considered as a target for gene therapy. More interestingly, we found that nuclear FHIT expression could be used as a good marker to distinguish PSA from Ca-ex-PA.*

Correspondence to: Dr. Omar Kujan, Ph.D., MSc, DDS, Head department of Biomedical Sciences, School of Dentistry, AlFarabi College, Riyadh, Saudi Arabia, P.O.Box: 85184. Tel: +966 501158867, e-mail: omar.kujan@gmail.com

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Pleomorphic salivary adenoma (PSA) is the most common neoplasm of salivary glands (1) and has been shown to occasionally undergo malignant transformation in its natural course (2). Carcinoma ex-pleomorphic adenoma (Ca-ex-PA) is considered to be a malignant transformation product of pre-existing pleomorphic adenoma (3-6). Ca-ex-PA is the most common malignant "mixed" tumour and has been estimated to account for 5-15% of all salivary gland malignancies (4-7). In fact, Ca-ex-PA is characterized by a variety of histological subtypes which exhibit different biological and prognostic behaviour (5, 8). Malignant transformation of human tumours requires a series of events of genetic alterations involving tumour suppressor genes, oncogenes, and chromosomal abnormalities (9, 10). The pathogenetic mechanisms involved in the progression of pleomorphic adenoma to a carcinoma remain unclear, requiring evaluation of molecular events in both pleomorphic adenoma and carcinoma arising from pleomorphic adenomas (11, 12). The current studies of the molecular biology of cancer have demonstrated that the loss of function of tumour suppressor genes such as *p53, CDKN2A, pRb, FHIT* may

lead to the development of many different cancer types (10, 13). The degree of inactivation of these genes appears to have impact on the tumours biological behaviour and prognosis (14, 15). The published literature on tumour markers in Ca-ex-PA is limited due to the fact that these tumours are rare. Our study aimed to characterize the alterations in the expression pattern of the protein products of genes controlling the cell cycle (*CDKN2A*) and apoptosis (*FHIT*), and to evaluate whatever inactivation of tumour suppressor genes increases with the tumour progression from normal salivary tissue to PSA and eventually Ca-ex-PA.

Materials and Methods

Case selection. A selected series of 29 cases of PSA, and 26 cases of Ca-ex-PA were retrieved from the archives of the Oral Pathology Departments in the North West region of England. Normal tissue of the salivary gland surrounding the tumour was used as a control in 29 cases of PSA. The immunohistochemical expression of antibodies against FHIT and CDKN2A was examined in the selected cases. The proposed criteria for defining Ca-ex-PA by Nagao *et al*. were used to select and reclassify our cases of Ca-ex-PA (16)

Inclusion criteria for Ca-ex-PA. Major gland primary lesion (parotid or submandibular); co-existent benign and malignant elements: Benign elements of: pleomorphic adenoma within the tumour mass; recognisable PSA ghost; extensive scar tissue; biopsy-proven history of previous PSA; malignant elements of: undifferentiated carcinoma; adenocarcinoma; evidence of vascular invasion, neural invasion, mitotic rate >5/(HPF), atypical cells in capsule, invasion of adjacent tissue; multiple patterns of differentiation including undifferentiated or adenocarcinoma patterns.

Exclusion criteria for Ca-ex-PA. Salivary carcinomas of uncertain type or classified other than carcinoma in PSA; carcinomas arising in PSA with a purely mucoepidermoid, adenoid cystic or other recognised pattern in the WHO classification (3), carcino-sarcoma, carcinoma *in situ* (transforming) PSA, secondary carcinoma occurring in or adjacent to the major glands. All specimens using hematoxylin and eosin slides were reviewed by two pathologists to confirm the histopathological diagnosis and to reclassify the included cases. The carcinoma cases were classified according to the above criteria as undifferentiated carcinoma or adenocarcinoma. The local Research Ethics Committee provided a favourable ethical opinion (Ref: 02/104 University of Manchester Ethics Committee).

Immunohistochemistry. Paraffin-embedded, 4-μm-thick tissue sections from all 55 specimens were cut. The sections were deparaffinized in xylene and rehydrated through graded alcohols. Sections were processed using the streptavidin-biotin-peroxidase method. Briefly, endogenous peroxidase was blocked by 3% hydrogen peroxidase for 5 min followed by tris-buffered saline (TBS) wash. Nonspecific immunoreactivity was blocked by incubation with normal goat serum for 20 min. A primary mouse monoclonal anti recombinant protein of mouse origin CDKN2A (Santa-Cruz Biotechnology, Heidelberg, Germany) was diluted to 4 μg/ml in 20 μg/ml TBS containing 0.1% bovine serum albumin for 1 h at room temperature. All sections were washed by TBS for 5 min. Sections were incubated with the biotinylated secondary antibody reagent for 30 min followed by TBS wash for 5 min. Slides were incubated with streptavidin and horseradish peroxidase for 30 min followed by TBS wash for 5 min. Incubate with a prepared chromogenic substrate solution (diaminobenzidine) for 15 min. Sections were counterstained with 0.25% methyl green in distilled water for 5 min then dehydrated and mounted in Depax. Squamous cell carcinoma was used as positive control. Negative control used substitution of the primary antibody with TBS. The percentage of CDKN2A-positive nuclei was semiquantively assessed by two independent observers and scored as: negative, 0, no expression of nuclear protein; 1, weak 0-25% of the total cells exhibit positive staining in the nucleus; 2, moderate staining 26-75% of the total cells in the test area show positive nuclear staining; 3, strong staining76-100% cells with positive nuclear staining. For staining sections with antibodies to FHIT, we followed our previously published protocol (17). FHIT immunoreactivity was categorized as 'negative' or 'low expression' (no staining, or immunoreactivity present in <10% of tumour cells) or 'positive' (immunoreactivity in ≥10% of tumour cells).

Statistical analysis. Only cells of the carcinomatous component of the Ca-ex-PA cases were scored, while the epithelial and mesenchymal cells of PSA were scored. For simplifying the

Table I. *Clinical and immunohistochemical characterization of FHIT, and CDKN2A in pleomorphic adenomas. M: Male; F: Female; +, >25% positively stained cells; –, ≤25% positively stained cells.*

Case no.	Age (Years)	Gender	FHIT	CDKN2A
1	87	F	+	+
2	52	M	+	–
3	63	M	+	+
4	48	F	+	–
5	76	F	+	+
6	47	M	+	+
7	62	F	+	+
8	33	M	+	+
9	49	F	+	–
10	45	M	+	+
11	63	F	+	+
12	53	F	+	–
13	27	F	+	–
14	59	F	+	+
15	33	F	+	+
16	55	F	+	–
17	26	F	+	+
18	65	F	+	+
19	40	M	+	+
20	57	M	+	+
21	34	M	+	+
22	74	F	+	–
23	67	F	+	–
24	32	M	+	–
25	31	M	+	–
26	62	F	+	+
27	76	F	+	+
28	21	F	+	–
29	61	F	+	–

Table II. *Clinical, histopathological and immunohistochemical characterization of FHIT and CDKN2A in carcinoma ex-pleomorphic adenomas. M: Male; F: Female; +, >25% positively stained cells; –, ≤25% positively stained cells.*

Case no.	Age (Years)	Gender	Gland	Histological subtype	Metastasis to lymph nodes*	FHIT	CDKN2A
1	77	F	Parotid	Adenocarcinoma NOS	Yes	–	–
2	28	M	Parotid	Adenocarcinoma NOS	No	+	+
3	78	M	Submandibular	Undifferentiated	Yes	+	+
4	45	M	Parotid	Undifferentiated	Yes	+	–
5	76	F	Parotid	Undifferentiated	No	+	+
6	82	F	Parotid	Undifferentiated	No	+	+
7	71	M	Parotid	Adenocarcinoma NOS	No	+	–
8	67	M	Submandibular	Undifferentiated	Yes	+	–
9	63	M	Submandibular	Undifferentiated	Yes	+	+
10	55	M	Submandibular	Undifferentiated	Yes	–	+
11	73	M	Parotid	Undifferentiated	Yes	+	+
12	71	M	Parotid	Undifferentiated	No	–	–
13	64	M	Parotid	Undifferentiated	Yes	+	+
14	60	F	Parotid	Undifferentiated	Yes	+	+
15	49	F	Submandibular	Undifferentiated	No	+	–
16	39	F	Parotid	Undifferentiated	Yes	–	+
17	56	M	Parotid	Undifferentiated	No	–	–
18	45	F	Parotid	Undifferentiated	Yes	+	+
19	57	M	Parotid	Undifferentiated	Yes	+	+
20	66	F	Parotid	Undifferentiated	No	–	+
21	86	F	Submandibular	Undifferentiated	Yes	+	+
22	17	F	Parotid	Undifferentiated	No	+	+
23	78	M	Submandibular	Undifferentiated	Yes	+	+
24	26	M	Parotid	Undifferentiated	No	+	–
25	31	F	Parotid	Undifferentiated	No	+	+
26	71	M	Parotid	Undifferentiated	No	+	+

*Metastasis to lymph nodes at the time of tumour resection.

statistical analysis, strong and moderate nuclear staining grouped as 'positive', and the negative and weak nuclear staining as 'negative'. The statistical analysis included the use of descriptive statistics, frequencies/proportions and crossed tabulation. Statistical analyses, including Mann–Whitney and Wilcoxon's nonparametric tests, were also performed on the data. All statistical tests were two-sided and *p*-values less than 0.05 were considered to be statistically significant.

Results

The clinical, histopathological and immunohistochemical features of the 55 tumours are summarized in Tables I and II. In the group of Ca-ex-PA, there were 15 males and 11 females ranging in age from 17 to 86 years, with a mean age of 55.9 years (SD±18.8 years). Nineteen tumours arose in the parotid gland (73.1%) and seven in the submandibular gland (26.9%). On the other hand, the mean age of patients with PSA was 51.7 (SD±17.2) years and females were more prevalent than males (1.9:1). Strong nuclear FHIT expression was found in all normal parotid cases (Figure 1A). Likewise, CDKN2A expression was positive in 28 (96.6%) cases out of 29, and negative in one (3.4%) case. Interestingly, nuclear FHIT expression was found in all PSA cases and most Ca-ex-PA cases (20/26; 76.9% Figure 1B). Only 6 out of 26 Ca-ex-PA cases exhibited loss of nuclear FHIT expression in cancerous cells (Figure 1C). Moreover, nuclear staining for CDKN2A was negative in 12/29(41.4%) of the PSA acinar and ductal cells, whereas 17/29 PSA cases (58.6%) had positive staining. Nuclear staining for CDKN2A was lost in 8/26 (30.8%) of the Ca-ex-PA cases and 18/26 (69.2%) had positive nuclear staining (Figure 1D). A significant difference (p=0.001) in the expression of CDKN2A between normal salivary parenchymal cells and PSA tumour cells was found, whereas there was no difference at all in FHIT expression (Table III). Interestingly, the expression pattern of FHIT was significantly different between the cases of PSA and those of Ca-ex-PA (p=0.014), while the expression of CDKN2A in Ca-ex-PA was no different from that in PSAs (Table III).

Discussion

Ca-ex-PA is a relatively uncommon salivary gland malignancy and presents a challenge in terms of diagnosis, treatment and prognosis. Better understanding of its

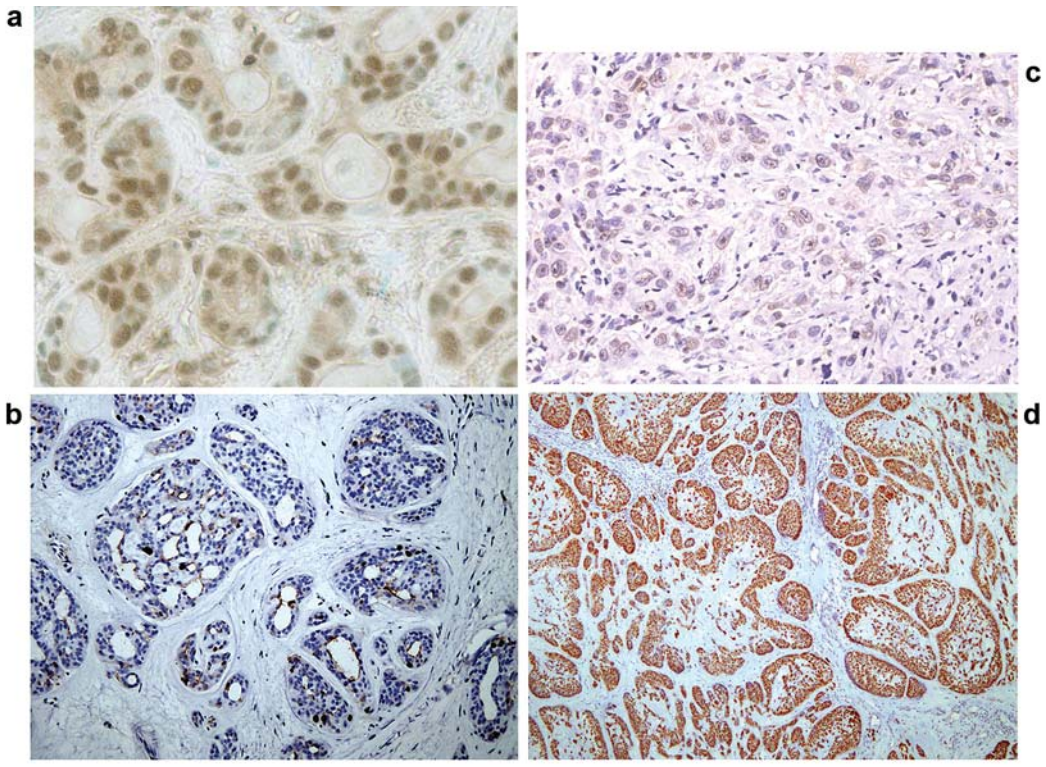

Figure 1. *Immunohistochemical expression of FHIT and CDKN2A in pleomorphic adenoma and carcinoma ex pleomorphic adenoma. A) Positive expression of FHIT in normal salivary gland (×400); B) Positive expression of FHIT in Ca-ex-PA (×100); C) Negative expression of FHIT in Ca-ex-PA (×400); D) Positive expression of CDKN2A in Ca-ex-PA (×200).*

Table III. *p-Values for comparison of the tumour markers in normal salivary parenchyma, pleomorphic adenoma and carcinoma ex-pleomorphic adenoma.*

	p-Value (Wilcoxon ranks test)	
Marker	Normal salivary parenchyma *vs*. pleomorphic adenoma	Pleomorphic adenoma cells *vs*. Carcinoma-ex-pleomorphic adenoma cells
FHIT	1.000	0.014
CDKN2A	0.001	0.564

biological and molecular profile would provide valuable information in this regard (5, 18). In fact, the development and progression of malignant tumours, including those of salivary origin, has proven to be a long-term, multistep process that involves accumulation of various genetic alterations. Tumour suppressor genes constitute a significant group of genes that play an important role in carcinogenesis (9, 13). The level of expression of these genes or their protein products has also been shown to have a prognostic or predictive value (15). Immunohistochemistry has proven to be a useful tool in daily diagnostic work to evaluate diagnostic and prognostic features of salivary gland tumours (19). In this study, we attempted to determine the immunohistochemical expression of two tumour suppressor genes (*FHIT* and *CDKN2A*) in normal salivary parenchyma, PSA and Ca-ex-PA and their role in the genesis of these tumours. We were also interested to assess whether these markers have a role in the prediction of malignant transformation model from PSA to Ca-ex-PA. In our series, clinical characteristics such as age, gender, and type of affected salivary gland were similar to those reported in the literature (3, 11, 20). Our previous data demonstrated that FHIT has been shown to play an important role in the malignant transformation of oral epithelial dysplasia into

squamous cell carcinoma (17). We also demonstrated a reproducible unique nuclear localisation pattern of FHIT in salivary parenchyma. This the first study to report on the immunostaining of FHIT in cases of Ca-ex-PA. The present study showed nuclear staining of FHIT in all cases of normal salivary parenchyma, PSA and in considerable fraction of Ca-ex-PA cases. In the English language literature, there are three studies that reported on the FHIT status in salivary gland tumours (21-23). All authors demonstrated *FHIT* gene alterations in PSA and malignant salivary gland tumours using cytogentics methods. Nagel *et al*. found aberrant FHIT transcripts in one of 38 normal salivary glands, three of 28 adenomas, and two of 16 carcinomas (22). There were no carcinoma ex pleomorphic adenoma cases included in their study group. In fact, immunohistochemistry is a useful method for showing loss of FHIT expression in tumours where FHIT may be inactivated by a variety of molecular mechanisms. Our present data show a loss of FHIT expression in 23.1% of all cases of Ca-ex-PA. This suggests that the loss of FHIT could be used as a marker for predicting the malignant transformation in PSA. In the current study, the expression of a member of the cyclin-dependent kinase inhibitor family (CDKN2A) was similar to that reported in the published literature for PSAs and Ca-ex-PAs (24-27). Interestingly, no statistically significant difference was found in the expression of this protein between PSAs and Ca-ex-PAs (p=0.564). The exact role played by each of the proteins analysed in the tumourigenesis in our series is not easy to define. However, the present study clearly demonstrated disruption of the cell cycle and apoptosis in cells of both PSA and Ca-ex-PA through the disruption of CDKN2A and FHIT. Moreover, our data demonstrated that CDKN2A is more likely to be disrupted through the pRb pathway, consistent with Etges *et al*. (25). It seems that the cumulative abnormalities of the regulatory network of retinoblastoma are one of the most frequent altered pathway in Ca-ex-PA. More interestingly, the present data indicated that FHIT inactivation is an event independent from the inactivation of other tumour suppressor markers (p=0.485). Without doubt, further research in cell biology is needed to improve the understanding of the interaction between these genes in malignant mixed salivary tumours. The identification of the subtypes of Ca-ex-PA is important due to the prognostic implications (11, 28). However, in our series, we thought that the correlation between the immunostaining characteristics of FHIT and CDKN2A and the histological subtypes would not be useful to identify the Ca-ex-PA subtypes as 88.5% (23/26) of our Ca-ex-PA series were subtyped as undifferentiated. A larger sample with statistically balanced distribution of Ca-ex-PA cases from all subtypes is required to generate a statistically significant distinction. Unfortunately, we were unable to determine the prognostic value of our studied markers because of the absence of the clinical follow up data. Nevertheless, our data identified CDKN2A alteration as an early event in the malignant transformation of PSA and could possibly be targeted for gene therapy. More interestingly, we found that nuclear expression of FHIT might be useful as a good marker to distinguish PSA from Ca-ex-PA.

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