

# The proliferation marker Chromatin Assembly Factor-1 is of clinical value in predicting the biological behaviour of salivary gland tumours

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**Abstract.** Salivary gland tumours (SGT) constitute a diagnostically challenging group of neoplasms with frequently unpredictable clinical outcome. The proliferation rate facilitates the identification of aggressive SGT. The Chromatin Assembly Factor-1 (CAF-1) is a major epigenetic regulator of nuclear chromatin organization during DNA replication. It plays a critical function in human tumourigenesis and has been proposed as a new proliferation and prognostic marker for some malignancies. This study focused on the role of CAF-1/p60 protein as a marker of clinical value for SGT. The expression of CAF-1/p60 was evaluated by immunohistochemistry on a retrospective series of 362 surgically excised benign and malignant SGT with different histogenesis and, when available, on fine-needle pre-surgical cytological biopsies. The resulting data were compared with traditional prognostic parameters, including the expression of the routine proliferation marker ki67/MIB1. CAF-1/p60 was detectable in all SGT, with highest degree of expression in metastasizing malignant tumours. Moreover, the cases of benign tumours which progressed to carcinoma during the follow-up, showed significantly higher CAF-1/p60 expression than non-

progressing benign SGT, both on histological sections and cytological smears of the primary tumour. Cox's multiple regression analysis selected CAF-1/p60 expression as the best independent predictor of cancer development for benign SGT ( $p < 0.0001$ ), and the best independent predictor of metastasis onset for malignant tumours ( $p < 0.0004$ ). Overexpression of CAF-1/p60, on histological and/or cytological samples, characterizes malignant SGT with aggressive behaviour, irrespective of their specific histotype, and allows the early diagnosis of progression toward malignancy of morphologically benign tumours.

## Introduction

Salivary gland tumours (SGT) constitute morphologically and biologically heterogeneous neoplasms, which give rise to significant diagnostic and management challenges (1-3). These tumours are rare, with an overall incidence of 0.5-3.0 cases per 100000 per year. Malignant SGT accounts for about 0.5% of all malignancies and for 3-6% of head and neck cancers (1).

Many studies attempted to better define the prognosis of these tumours (4-6). However, the available markers are unable to ultimately discriminate, among benign SGT, the cases that will give rise to progression toward malignancy, and, for malignant SGT, the cases with a metastasizing behaviour.

Cell proliferation rate has been proposed as an useful adjunctive tool for predicting the outcome of SGT, but there is not a definitive agreement between researchers on the best proliferation-associated prognostic marker for these tumours (3,4,6-12).

The orderly progression of the cell cycle is governed by epigenetic modifications, mostly concerning the hierarchical assembly and remodelling of chromatin, which are regulated

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**Key words:** Chromatin Assembly Factor-1, salivary gland tumours, aggressive behaviour

Table I. Clinical and pathological features, CAF-1/p60 and ki67/MIB1 immunohistochemical expression in 268 cases of benign SGT.

Characteristics	Total	(%)	CAF-1/p60 (quickscore)		ki67/MIB1 (quickscore)		
			5 (2+3)	6 (3+3)	3 (1+2)	4 (2+2)	5 (2+3)
No. of subjects							
Male	116	43.28	114 (42.53)	2 (0.75)	100 (37.31)	8 (2.99)	8 (2.99)
Female	152	56.72	150 (55.97)	2 (0.75)	131 (48.69)	12 (4.48)	9 (3.36)
Age							
Average (range)	39.83	(9-80)	-	-	-	-	-
Disease site							
Parotid gland	203	75.75	200 (74.63)	3 (1.12)	180 (67.16)	12 (4.48)	11 (4.11)
Submandibular gland	33	12.31	33 (12.31)	0	30 (11.20)	2 (0.75)	1 (0.37)
Sublingual gland	1	0.4	1 (0.37)	0	1 (0.37)	0	0
Minor salivary gland	31	11.57	30 (11.20)	1 (0.37)	20 (7.46)	6 (2.23)	5 (1.87)
Histotype							
BCA	3	1.12	3 (1.12)	0	3 (1.12)	0	0
PA	261	97.39	258 (96.27)	3 (1.12)	226 (84.33)	19 (7.09)	16 (5.97)
Atypical PA	4	1.49	3 (1.12)	1 (0.37)	2 (0.75)	1 (0.37)	1 (0.37)
Evolution to carcinoma ex-PA							
No	263	98.13	263 (98.14)	0	230 (85.82)	18 (6.72)	15 (5.59)
Yes	5	1.87	1 (0.37)	4 (1.49)	1 (0.37)	2 (0.75)	2 (0.75)

by various Assembly Factors (13,14). Among these, the Chromatin Assembly Factor-1 (CAF-1), a heterotrimeric complex, composed of the histone-chaperone proteins p48, p60 and p150, is the most powerful discriminator between the proliferative and the quiescent state (15-17). CAF-1/p60 has been recently proposed as a new sensitive marker of cell proliferation, able to predict the prognosis of some human cancer types (18-20).

We have performed a study on a retrospective series of benign and malignant SGT, with the aim to establish whether the immunohistochemical expression of CAF-1/p60 protein may have a value in discriminating SGT with different clinical behaviour, beyond the available prognostic parameters including the routinely assessed proliferation marker ki67/MIB1.

## Materials and methods

**Selection of cases.** The study population was selected among the patients diagnosed with SGT at the Federico II University of Naples, Italy, from April 1993 to March 2008.

Only the cases of patients treated primarily with surgery, with healthy surgical margins distant from the tumour by at least 1 cm, and with clinical follow-up data not less than 12 months, were considered for the study.

Patients characteristics, the anatomic site and histotype of primary tumours and, for malignant SGT, the degree of differentiation and tumour stage were collected and recorded in a data base (21,22).

**Immunohistochemistry.** Immunohistochemical analysis was performed on 4- $\mu$ m thick serial sections, mounted on poly-L-lysine coated glass slides. Sections were deparaffinized, and underwent antigen retrieval by microwave oven treatment (5 min x 3 times, in 1% sodium citrate buffer, pH 6.0); non-specific bindings were blocked by incubation (2 h at room temperature) with 1.5 % non-immune mouse serum (1:20, Dakopatts, Hamburg, Germany). Endogenous peroxidases were quenched with 0.3% hydrogen peroxide in methanol; slides were rinsed twice with Tris-HCl buffer, and incubated overnight at 4°C with anti-CAF-1/p60 antibody (SS53-ab8133, Abcam, Cambridge, MA, USA, diluted 1:300) and ki67/MIB1 antibody (MIB1, Dako A/S, Glostrup, Denmark, diluted 1:200) (19,20,23).

The standard streptavidin-biotin linked horseradish peroxidase (LSAB) technique using the Dako (LSAB kit HRP, Carpinteria, CA) was then performed, with the 3,3'-diaminobenzidine (DAB, Vector Laboratories Inc., Burlingame, CA, USA) being used as a substrate chromogen solution for the development of the peroxidase activity. After nuclear counterstaining with Mayer's haematoxylin for 30 sec, sections were mounted and cover-slipped with a synthetic medium.

For each run, positive controls were performed on sections of breast carcinomas for CAF-1/p60, and on small bowel samples for ki67/MIB1. For negative controls, non-immune serum in TBS buffer (1:500) was used instead of the two primary antibodies. The cells with a definite brown nuclear staining were judged as positive for both the anti-

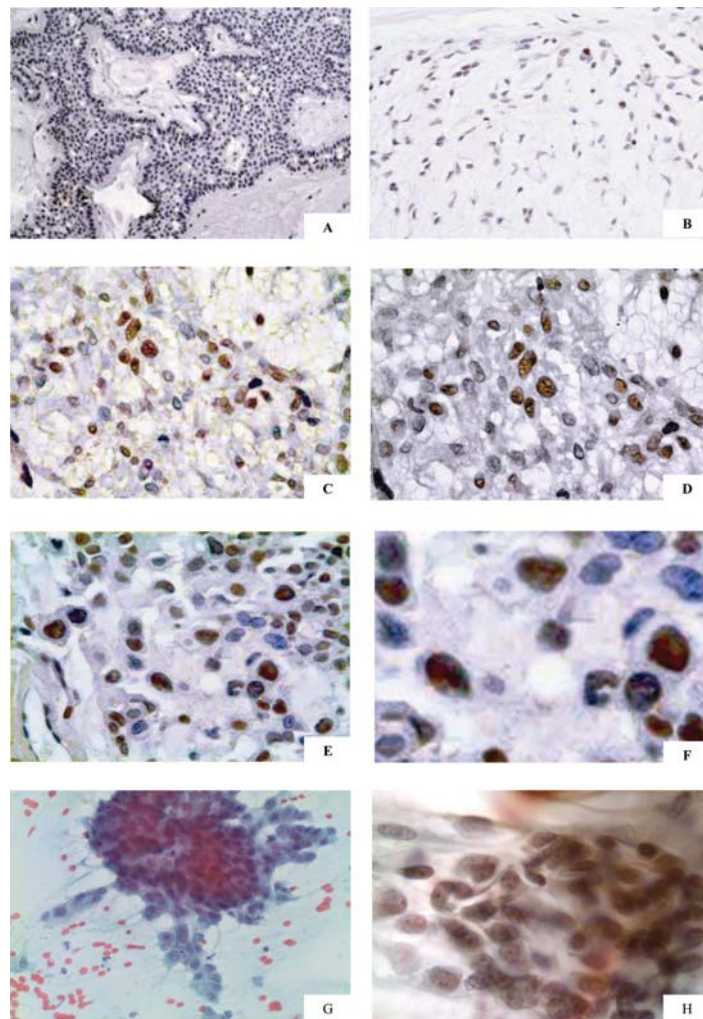


Figure 1. Immunohistochemical expression of CAF-1/p60 in benign SGT (LSAB-HRP, nuclear counterstaining with haematoxylin). Focal and weak nuclear immunostaining for CAF-1/p60 in BCA (A, x106) and PA (B, x150). Higher expression of CAF-1/p60 in two cases of PA (ordinary type: C, x200; D, x250 and atypical: E, x250; F, x400) which progressed to CXPA during follow-up; PA progressed to CXPA (PAP-stained FNAB: G, x250; CAF-1/p60 immunostaining: H, x400).

bodies. The immunohistochemical expression was evaluated semi-quantitatively and scored according to the 'quickscore' method, which takes into consideration either the percentage of immunostained neoplastic cells throughout the entire tumour section (scores 1-6: 1, 0-4%; 2, 5-19%; 3, 20-39%; 4, 40-59%; 5, 60-79%; 6, 80-100%), termed 'category A', and the staining intensity (scores 0-3, corresponding to negative, weak, intermediate and strong staining), termed 'category B' (24).

The evaluation was performed independently by two expert pathologists (S.S. and M.M.), unaware of patients' clinical status. Discordant cases were discussed and resolved by consensus.

For 23 cases of benign tumours, pre-surgical PAP-stained slides of the fine-needle aspiration biopsy (FNAB) cytological samples, used for morphological diagnosis, were also available, and were re-used for the immunostaining with CAF-1/p60 protein, according to the technique described in literature (Table I) (25).

**Statistical analysis.** Data were analyzed with SPSS package for Windows (release 13.0). The intra- and inter-observer agreement of the evaluation of the CAF-1/p60 and ki67/

MIB1 quickscore was calculated by k-statistics: a K coefficient  $>0.75$  indicated excellent reproducibility; a K-value between 0.4 and 0.75 denoted moderate reproducibility, and a K-value  $<0.4$  denoted marginal or poor reproducibility (26).

To evaluate the prognostic value of CAF-1/p60 expression with respect to conventional parameters and ki67/MIB1 expression, a univariate analysis was performed by Kaplan-Meier plots for each variable. The significant level was set at  $p < 0.05$ . To verify whether variables were univariately related to our end-points (development of malignant tumour in patients with benign cancer baseline and disease-free interval in patients with malignant tumours baseline) curves were compared by the log-rank test or the t-test for trend, when two or three curves had to be compared, respectively.

In order to establish whether CAF-1/p60 expression contributed to a better definition of the prognosis of patients, after the conventional data had been taken into account, a multiple regression analysis according to the Cox's proportional hazard model was performed.

The clinical usefulness of prognostic indices predicting the risk for cancer development in patients who had a

Table II. Clinical and pathological features, CAF-1/p60 and ki67/MIB1 immunohistochemical expression in 94 cases of malignant SGT.

Patient no.	Gender	Age	Diagnosis	Gland	CAF-1/p60 (quickscore)	ki67/MIB1 (quickscore)	F-UP	Stage
1	M	49	PLGC	Minor salivary gland	6 (3+3)	3 (1+2)	26	pT1N0M0
2	F	54	PLGC	Parotid	8 (5+3)	3 (1+2)	60 R (34)	pT1NxM0
3	F	13	AC	Parotid	7 (4+3)	4 (2+2)	74	pT1NxM0
4	F	33	AC	Parotid	6 (3+3)	4 (2+2)	67	pT3N0M0
5	M	45	AC	Parotid	7 (4+3)	4 (2+2)	70	pT2NxM0
6	F	47	AC	Parotid	6 (3+3)	4 (2+2)	42	pT1NxM0
7	F	78	AC	Minor salivary gland	7 (4+3)	4 (2+2)	44	pT3NxM0
8	F	41	AC	Parotid	7 (4+3)	4 (2+2)	20	pT1N0M0
9	F	57	AC	Parotid	6 (3+3)	4 (2+2)	93	pT1NxM0
10	M	52	AC	Parotid	8 (5+3)	4 (2+2)	150 R (40,142)	pT1NxM0
11	M	55	AC	Parotid	6 (3+3)	4 (2+2)	158	pT1N0M0
12	F	19	AC	Submandibolar gland	6 (3+3)	4 (2+2)	142	pT1NxM0
13	F	57	AC	Parotid	6 (3+3)	4 (2+2)	180	pT2NxM0
14	M	50	AC	Parotid	6 (3+3)	4 (2+2)	110	pT2NxM0
15	F	17	AC	Parotid	7 (4+3)	4 (2+2)	167	pT1NxM0
16	M	60	AC	Parotid	7 (4+3)	4 (2+2)	192	pT2NxM0
17	F	23	ACC	Submandibolar gland	7 (4+3)	4 (2+2)	97	pT1NxM0
18	F	34	ACC	Minor salivary gland	7 (4+3)	5 (2+3)	82	pT1NxM0
19	F	39	ACC	Parotid	6 (3+3)	6 (3+3)	29	pT2N0M0
20	F	41	ACC	Submandibolar gland	7 (4+3)	6 (3+3)	54	pT3N0M0
21	M	48	ACC	Minor salivary gland	9 (6+3)	6 (3+3)	71 M (58) R (33)	pT4aNxM0
22	F	56	ACC	Minor salivary gland	8 (5+3)	6 (3+3)	34	pT4bNxM1
23	F	63	ACC	Minor salivary gland	7 (4+3)	4 (2+2)	44	pT2NxM0
24	F	41	ACC	Submandibolar gland	6 (3+3)	4 (2+2)	141	pT3N0M0
25	F	74	ACC	Minor salivary gland	6 (3+3)	4 (2+2)	181	pT2NxM0
26	F	49	ACC	Submandibolar gland	7 (4+3)	5 (2+3)	12	pT2N0M0
27	F	49	ACC	Minor salivary gland	7 (4+3)	6 (3+3)	173	pT2NxM0
28	F	69	ACC	Minor salivary gland	7 (4+3)	6 (3+3)	84	pT2NxM0
29	F	48	ACC	Minor salivary gland	8 (5+3)	6 (3+3)	193 R (61)	pT1NxM0
30	M	63	ACC	Minor salivary gland	6 (3+3)	5 (2+3)	41	pT4aNxM0
31	M	49	ACC	Minor salivary gland	6 (3+3)	6 (3+3)	170	pT1NxM0
32	M	59	ACC	Minor salivary gland	8 (5+3)	5 (2+3)	185	pT2N0M0
33	M	69	ACC	Minor salivary gland	6 (3+3)	6 (3+3)	16	pT1NxM0
34	F	56	ACC	Submandibolar gland	7 (4+3)	4 (2+2)	177	pT2NxM0
35	F	69	ACC	Minor salivary gland	7 (4+3)	4 (2+2)	181	pT1NxM0
36	M	50	ACC	Parotid	8 (5+3)	4 (2+2)	177	pT3N2bM0
37	F	33	ACC	Minor salivary gland	6 (3+3)	4 (2+2)	34	pT1NxM0
38	F	20	Low-grade MEC	Parotid	6 (3+3)	4 (2+2)	49	pT1N0M0
39	M	34	Low-grade MEC	Parotid	7 (4+3)	5 (2+3)	137	pT1N1M0
40	F	57	Low-grade MEC	Minor salivary gland	7 (4+3)	5 (2+3)	173	pT3N0M0
41	M	14	Low-grade MEC	Minor salivary gland	6 (3+3)	5 (2+3)	128	pT1NxM0
42	F	18	Low-grade MEC	Parotid	6 (3+3)	4 (2+2)	92	pT2N0M0
43	M	37	Low-grade MEC	Parotid	6 (3+3)	4 (2+2)	129	pT1NxM0
44	F	38	Low-grade MEC	Parotid	6 (3+3)	4 (2+2)	57	pT1N0M0
45	F	40	Low-grade MEC	Parotid	6 (3+3)	4 (2+2)	69	pT4aN0M0
46	F	42	Low-grade MEC	Parotid	7 (4+3)	4 (2+2)	127	pT1NxM0
47	M	55	Low-grade MEC	Minor salivary gland	7 (4+3)	4 (2+2)	107	pT1NxM0
48	F	62	Low-grade MEC	Minor salivary gland	7 (4+3)	5 (2+3)	103	pT3NxM0
49	M	74	Low-grade MEC	Minor salivary gland	6 (3+3)	5 (2+3)	129	pT1NxM0
50	M	63	Low-grade MEC	Minor salivary gland	6 (3+3)	5 (2+3)	33	pT1NxM0
51	F	60	Low-grade MEC	Minor salivary gland	7 (4+3)	5 (2+3)	12	pT1NxM0
52	F	49	Low-grade MEC	Minor salivary gland	7 (4+3)	5 (2+3)	12	pT1NxM0
53	F	42	Low-grade MEC	Parotid	6 (3+3)	5 (2+3)	128	pT1NxM0

Table II. Continued.

Patient no.	Gender	Age	Diagnosis	Gland	CAF-1/p60 (quickscore)	ki67/MIB1 (quickscore)	F-UP	Stage
54	F	40	Low-grade MEC	Minor salivary gland	7 (4+3)	5 (2+3)	162	pT2NxM0
55	M	39	Low-grade MEC	Minor salivary gland	6 (3+3)	5 (2+3)	199	pT1NxM0
56	F	37	Low-grade MEC	Minor salivary gland	6 (3+3)	5 (2+3)	20	pT1NxM0
57	M	49	Low-grade MEC	Parotid	7 (4+3)	5 (2+3)	96	pT1NxM0
58	F	64	Low-grade MEC	Minor salivary gland	7 (4+3)	5 (2+3)	18	pT1NxM0
59	F	33	Low-grade MEC	Minor salivary gland	7 (4+3)	5 (2+3)	197	pT1NxM0
60	M	39	Intermediate-grade MEC	Parotid	8 (5+3)	5 (2+3)	140 R (48)	pT2N0M0
61	F	51	Intermediate-grade MEC	Minor salivary gland	9 (6+3)	6 (3+3)	132 M (16)	pT2N2bM0
62	M	57	Intermediate-grade MEC	Minor salivary gland	8 (5+3)	6 (3+3)	94 R (12)	pT1NxM0
63	M	72	Intermediate-grade MEC	Minor salivary gland	7 (4+3)	6 (3+3)	31	pT1NxM0
64	F	74	Intermediate-grade MEC	Minor salivary gland	6 (3+3)	6 (3+3)	95	pT1NxM0
65	F	80	Intermediate-grade MEC	Minor salivary gland	6 (3+3)	6 (3+3)	97	pT3NxM0
66	M	26	Intermediate-grade MEC	Parotid	6 (3+3)	5 (2+3)	23	pT1N0M0
67	M	60	Intermediate-grade MEC	Parotid	6 (3+3)	5 (2+3)	34	pT2N0M0
68	M	50	Intermediate-grade MEC	Minor salivary gland	6 (3+3)	6 (3+3)	12	pT1NxM0
69	M	88	Intermediate-grade MEC	Minor salivary gland	7 (4+3)	6 (3+3)	12	pT1NxM0
70	M	77	Intermediate-grade MEC	Minor salivary gland	7 (4+3)	5 (3+2)	170	pT3N0M0
71	M	62	Intermediate-grade MEC	Minor salivary gland	7 (4+3)	5 (3+2)	45	pT1NxM0
72	F	54	Intermediate-grade MEC	Parotid	6 (3+3)	5 (3+2)	12	pT1NxM0
73	F	59	Intermediate-grade MEC	Parotid	6 (3+3)	5 (3+2)	14	pT1NxM0
74	M	56	High-grade MEC	Minor salivary gland	8 (5+3)	6 (3+3)	131 R (75)	pT2N0M0
75	F	32	High-grade MEC	Parotid	7 (4+3)	5 (2+3)	200	pT4NxM0
76	F	33	High-grade MEC	Minor salivary gland	7 (4+3)	5 (2+3)	23	pT1NxM0
77	F	37	High-grade MEC	Minor salivary gland	6 (3+3)	6 (3+3)	21	pT1NxM0
78	M	46	High-grade MEC	Minor salivary gland	8 (5+3)	6 (3+3)	126 M (13)	pT2N1M0
79	F	47	High-grade MEC	Minor salivary gland	7 (4+3)	6 (3+3)	23	pT2NxM0
80	M	51	High-grade MEC	Sublingual gland	7 (4+3)	6 (3+3)	72	pT2N0M0
81	F	58	High-grade MEC	Parotid	8 (5+3)	6 (3+3)	39 M (2)	pT3N2M0
82	M	60	High-grade MEC	Submandibular gland	6 (3+3)	6 (3+3)	117	pT3NxM0
83	F	41	High-grade MEC	Minor salivary gland	7 (4+3)	6 (3+3)	12	pT1NxM0
84	F	78	High-grade MEC	Sublingual gland	7 (4+3)	6 (3+3)	142	pT2NxM0
85	F	27	High-grade MEC	Minor salivary gland	7 (4+3)	6 (3+3)	155	pT1NxM0
86	M	60	High-grade MEC	Parotid	6 (3+3)	6 (3+3)	34	pT3N0M0
87	F	56	CXPA	Minor salivary gland	7 (4+3)	6 (3+3)	23	pT2NxM0
88	M	19	CXPA	Sublingual gland	7 (4+3)	7 (4+3)	26	pT2N0M0
89	F	24	CXPA	Parotid	7 (4+3)	7 (4+3)	97	pT2N0M0
90	M	42	CXPA	Parotid	8 (5+3)	7 (4+3)	55 R (10) N (10)	pT1N0M0
91	M	43	CXPA	Parotid	6 (3+3)	7 (4+3)	63	pT3N0M0
92	M	72	CXPA	Parotid	7 (4+3)	6 (3+3)	166	pT2NxM0
93	F	89	CXPA	Parotid	6 (3+3)	6 (3+3)	151	pT3NxM0
94	M	50	CXPA	Parotid	7 (4+3)	7 (4+3)	166	pT2N0M0

baseline benign tumour or the risk of an adverse event (recurrence or metastasis) in patients who had a baseline malignant tumour was calculated. Sensitivity and specificity of prognostic indices were computed according to standard formula. Receiver operating characteristic (ROC) curves were drawn and areas under the curve were calculated, to evaluate the discriminatory ability of each parameter, as follow: <0.7, no discrimination; 0.71-0.79, acceptable; 0.8-0.89, excellent;  $\geq 0.9$ , outstanding discrimination (27).

## Results

The selected study population consisted of a cohort of 362 SGT (268 benign and 94 malignant).

**Benign SGT.** Benign tumours were composed of 265 PA, 4 of which with atypical/'bizarre' cells, and 3 cases of monomorphic (basal cell) adenomas. The clinical features of patients are reported in Table I.

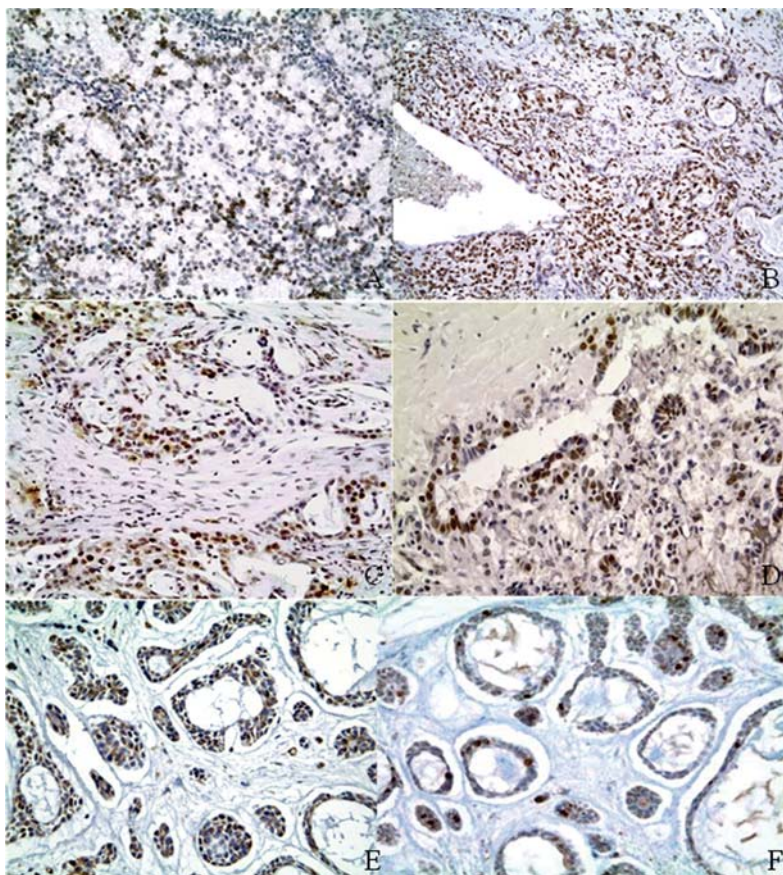


Figure 2. CAF-1/p60 overexpression in malignant SGT (LSAB-HRP, nuclear counterstaining with haematoxylin). Strong immunostaining for CAF-1/p60 in metastasizing malignant SGT: AC (A, x106), CXPA (B, x106), ME (C, x106), PLGA (D, x106). Metastasizing ACC: the same case showed strong expression for CAF-1/p60 (E, x150), and moderate expression for ki67/MIB1 (F, x150).

The cases for which PAP-stained slides were re-used for immunocytochemical evaluation of CAF-1/p60 were all PA (15 cases, parotid gland; 5 cases, submandibular gland; 3 cases, minor salivary glands, Fig. 1G).

During follow-up (mean 100.02 months, median 90.5 months, range 13-203 months), 5 patients (1.9%) developed a CXPA. In three cases, the baseline tumour was a PA of the parotid gland, and in the remaining two cases atypical PA of minor salivary glands.

**Malignant SGT.** The malignant SGT consisted of 21 cases of adenoid cystic carcinomas (ACC), 2 polymorphous-low grade adenocarcinomas (PLGC), 14 acinic cell carcinomas (AC), 49 muco-epidermoid carcinomas (MEC: 22 low-grade, 14 intermediate-grade and 13 high-grade tumours) and 8 cases of carcinoma ex-PA (CXPA). The clinical features of all the tumours are reported in Table II.

During follow-up (mean 92.56 months, median 92.5 months, range 12-200 months) 6 patients (6.38%) had recurrence of the tumour (1PLGC, 1AC, 1ACC, 3MEC), 1 (1.06%) recurrence and nodal metastasis (1CXPA), 1 (1.06%) recurrence and distant metastasis (1ACC) and 3 (3.19%) distant metastasis (3 MEC).

#### Immunohistochemistry

**CAF-1/p60 expression in benign SGT.** The large majority of benign SGT (264 cases, 98.51%) showed a quickscore 5 of

immunostaining for CAF-1/p60 (Fig. 1A and B). Only 4 cases (1.55%; all PA, two of the ordinary type and two atypical), which showed malignant transformation (CXPA) during follow-up, presented instead a quickscore 6 (Fig. 1C-F) (Table I).

The cytological smears concerned cases of PA which showed, on histological sections, a quickscore 5 in 20 cases and a score 6 in 3 cases which progressed to CXPA (Fig. 1G). Immunocytochemistry showed clusters of neoplastic cells strongly positive for the protein only in these 3 cases (Fig. 1H).

**ki67/MIB1 expression in benign SGT.** In the majority of benign SGT (231 out 268, 86.20%), a quickscore 3 of ki67/MIB1 expression was found; 20 cases (7.46%) showed a quickscore 4 and the remaining 17 cases (6.34%) a quickscore 5.

Among the cases which progressed toward malignancy during the follow-up, the quickscore was, respectively: 3 in 1 case, 4 in 2 cases and 5 in 2 cases (Table I).

**CAF-1/p60 expression in malignant SGT.** Overall, the quickscore for CAF-1/p60 expression ranged from 6 to 9 in malignant SGT (6-8 in AC, 6-9 in ACC, 6-7 in low-grade MEC, 6-9 in intermediate-grade MEC, 6-8 in high grade MEC, CXPA and PLGA).

The cases that recurred or progressed to nodal and/or distant metastases, showed always a quickscore 8-9 for CAF-1/p60 (Table II, Fig. 2A-E).

*ki67/MIB1 expression in malignant SGT.* The quickscore for ki67/MIB1 in malignant SGT ranged from 4 to 7 (AC: quickscore 4; ACC: 4-6; low-grade MEC: 4-5; intermediate- and high-grade MEC: 5-6; CXPA: 6-7; PLGC: 3). The recurrent and/or metastasizing cases showed a quickscore between 3 and 7 (Table II, Fig. 2F).

*Expression of CAF-1/p60 and ki67/MIB1 in normal salivary glands.* The residual normal salivary glands surrounding either benign and malignant SGT showed a quickscore 2 for ki67/MIB1 and 2-3 for CAF-1/p60.

#### Statistical analysis

*K-statistics.* The level of agreement of the evaluation of the quickscore of both CAF-1/p60 and ki67/MIB1, expressed by the K-coefficient, was 0.8 (excellent agreement) for both intra- and inter-observer evaluations, respectively.

*Factors predicting development of cancer in patients with benign tumour baseline.* According to univariate analysis, cancer onset was significantly related to: ki67/MIB1 expression ( $p < 0.0001$ ) and CAF-1/P60 ( $p < 0.0001$ ). Cox's multiple regression analysis selected CAF-1/p60 expression as the best independent and significant predictor of cancer development ( $p < 0.0001$ ).

*Factors predicting disease-free intervals in patients with malignant tumour baseline.* According to univariate analysis, the recurrence of tumour was significantly related to: female gender ( $p < 0.05$ ), ki67/MIB1 ( $p < 0.0002$ ) and CAF-1/p60 ( $p < 0.0001$ ). Applying Cox's multiple regression analysis, CAF-1/p60 expression ( $p < 0.002$ ) and staging ( $p < 0.001$ ) were the best set of covariates significantly predicting recurrence of the disease.

Concerning the occurrence of distant metastases, the univariate analysis showed that the expression of ki67/MIB1 ( $p < 0.001$ ), CAF-1/p60 ( $p < 0.0001$ ) and staging ( $p < 0.0001$ ) were significantly related to the metastasis development. Cox's multiple regression analysis selected CAF-1/p60 as the best independent predictor of metastasis onset ( $p < 0.0004$ ).

*Clinical usefulness of CAF-1/p60 index.* To further evaluate the usefulness of the CAF-1/p60 expression in the prediction of cancer development in patients who had a baseline benign tumour, sensitivity and specificity were calculated for each point of the quickscore, and receiver operating characteristic (ROC) curves were drawn. The best discriminant point of CAF-1/p60 quickscore was 5, which had 80% sensitivity and 100% specificity, and the calculated area under curve corresponded to 0.9 (Fig. 3).

A similar analysis was performed to evaluate the usefulness of CAF-1/p60 in the prediction of an adverse event (recurrence, or nodal or distant metastasis) in patients who had a baseline malignant tumour. Sensitivity and specificity in predicting both recurrence of disease and metastasis, were calculated for each point of the quickscore, and ROC curves were drawn. The best discriminative point of CAF-1/p60 quickscore predicting both recurrence of disease or metastasis risk was 7, which showed 100% sensitivity and 93% specificity in predicting recurrence of a tumour, and 100%

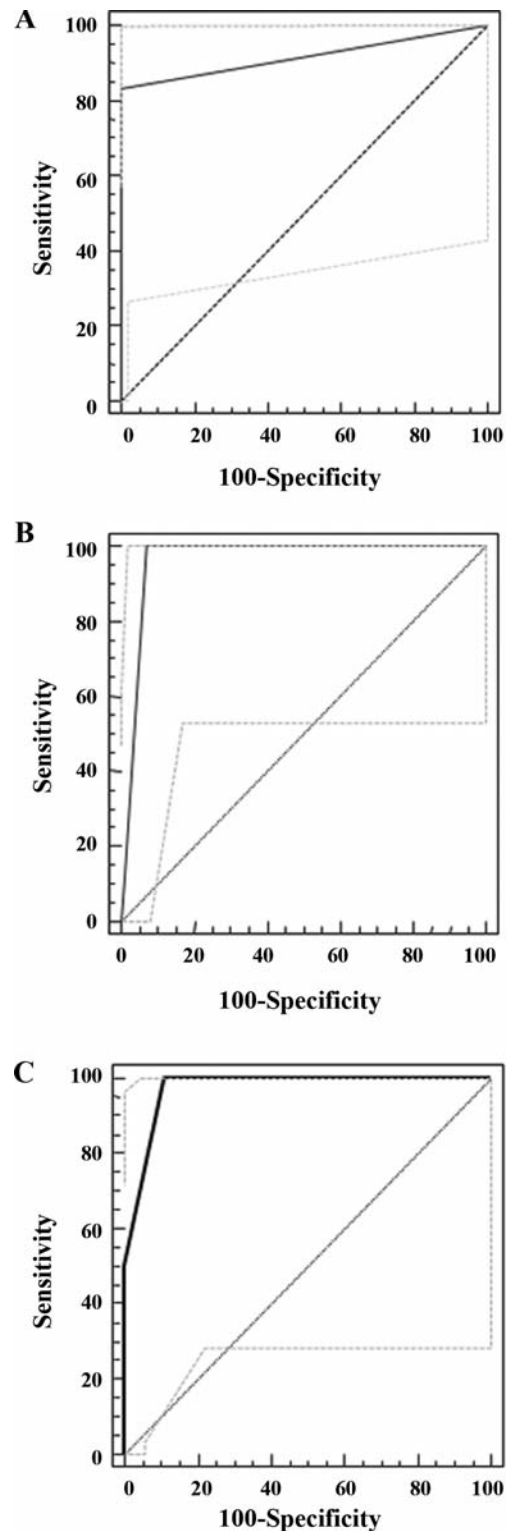


Figure 3. Receiver operating characteristic curves: (A) prediction of cancer development according to CAF-1/p60 immunopattern; (B) prediction of disease recurrence according to CAF-1/p60 immunopattern; (C) prediction of metastasis occurrence according to CAF-1/p60 immunopattern.

sensitivity and 89% specificity in predicting metastasis. Calculated areas under curve were 0.96 for the prediction of disease recurrence and 0.97 for prediction of metastasis, respectively (Fig. 3).

## Discussion

Salivary gland neoplasms comprise the most heterogeneous group of tumours of any body site, with frequent overlap of histological parameters and biological outcome (1,2,28,29). Efforts to identify biomarkers able to assist in the differential diagnosis of these tumours and in predicting their evolution did not provide conclusive results (29-31).

Recent studies evidenced that epigenetic alterations may play a role in the development and progression of SGT (32-35). However, a relatively limited variety of SGT histotypes and genes analyzed for epigenetic regulation has been examined (36). The assembly and remodelling of chromatin are among the most important epigenetic modifications, in eukaryotic cells (37).

CAF-1 promotes the first step of nucleosome assembly in replicating DNA. The expression of CAF-1/p60 subunit directly correlates with cell proliferation in normal and neoplastic cells. This seems particularly attractive for researchers facing with SGT, considering that cell proliferation is one of the few proposed prognostic parameters for most of these tumours. The large majority of reports on this topic concern the immunohistochemical evaluation of the proliferating cell compartment by the ki67/MIB1 antibody.

The methodological approaches to this evaluation differed considerably among researchers. In particular, the evaluated ki67/MIB1 stained cells ranged from 500 to 1000, randomly scored either with or without the aid of a video-scaler, or from the densest area of immunopositive cancer cells for mm<sup>2</sup> of tumour tissue (4,11,12).

These discrepancies and the frequently small retrospective series studied, due to the rarity of SGT, may explain the questionable results concerning the clinical value of ki67/MIB1-stained cell fraction in evaluating the biological behaviour of SGT.

A correlation between a high ki67/MIB1 labelling index and the unfavourable prognosis of MEC has been reported, but for ACC some studies found ki67/MIB1 useful for predicting the biologic behaviour and/or treatment failure, while others found it not associated with morphology or clinical course of patients (6,10,11,38).

Moreover, for AC, a study showed that most patients with tumour ki67/MIB1 indices above 10% had unfavourable outcomes, whereas other reports evidenced overlap of indices between favourable and worse outcomes or showed that ki67/MIB1 revealed of statistical value in predicting tumour outcome (7,39).

For PLGA, the ki67/MIB1 index was generally not found useful in predicting clinical behaviour and, in particular, Lazzaro and Cleveland showed that for PA, PLGA and ACC, the ki67/MIB1 score is not related either to the benign or malignant nature of tumours or to their specific clinical course (6,40-42).

For benign SGT, it has been stated that a moderate mitotic activity and older patient age, large tumour size and the occurrence in the submandibular gland, are associated to a greater likelihood of malignant transformation (43). However, a recent study evidenced that proliferation markers did not reveal significant differences between morpho-

logically benign metastasizing PA and a control group of conventional PA (44).

Finally, the ki67/MIB1 index was thought either of overall utility in predicting the clinical behaviour of SGT, or it was considered significant only when coupled to TUNEL evaluation and p53 staining (4,12,45).

Therefore, as it has recently emerged also for other malignant tumours, unresolved questions concerning the role of ki67/MIB1 as a real independent marker of prognosis for the major histotypes of malignant SGT still need to be answered.

Moreover, it has to be clarified whether this proliferation marker has the potential to detect early the cases of benign tumours which are trans-differentiating toward a malignant phenotype. It is still uncertain whether ki67/MIB1 is the best proliferation-associated predictor of malignancy and aggressiveness for SGT.

In this study, we decided to score an entire histological section of each SGT for ki67/MIB1 and CAF-1/p60 expression, evaluating them according to the 'quickscore' method, which is easy to perform by pathologists in addition to the routine diagnostic analysis. We found CAF-1/p60 always expressed, in benign as in malignant SGT, with the highest degree of overexpression in more aggressive cases.

This was in-line with the reported association between the hyper-expression of the protein and the deregulation of the cell proliferation in malignant tumours of breast, tongue and prostate and in malignant melanoma, and was extremely significant in terms of statistical evaluation, besides tumour histotype, grade and stage at diagnosis (18,20).

The overexpression of CAF-1/p60 may be then considered an overall hallmark of malignancy for SGT, which may assist in the challenging differential diagnosis between benign and malignant SGT. Moreover, the protein shows further significance in discriminating the cases of malignant SGT with metastatic ability.

Interesting considerations emerge also by the finding, in the majority of PA followed by the occurrence of CXPA after surgery, of a CAF-1/p60 quickscore higher than in non-progressed benign SGT, with aggregates of neoplastic cells strongly positive for CAF-1/p60 in the corresponding FNAB preoperative samples of some of these cases.

In our opinion, CAF-1/p60 is highly effective to early identify the cases of benign epithelial salivary gland tumours which are trans-differentiating toward a malignant phenotype. This sounds particularly attractive for the evaluation of the biological behaviour of the clinically benign PA with 'bizarre/atypical' features, which sometimes may show an aggressive biological course, and of even greater importance for the cases of the classical form of PA, which rarely may evolve toward malignancy, in a manner unpredictable upon the classical histological and cytological parameters (46-48).

To date, this is the first study addressing the role of the expression of CAF-1/p60 in predicting the outcome of patients with SGT.

Considering the rarity of these tumours, we believe that our single institution's series of cases, for number, histotype of lesions and length of follow-up, may be considered adequate to evaluate the biological significance of the expression of CAF-1/p60 in SGT.

Our results emphasize the role of epigenetic changes in SGT, and evidence a pivotal role for CAF-1/p60 as a marker of aggressiveness, with an additional value with respect to ki67/MIB1 in predicting the outcome of malignant SGT and improving the chances of early diagnosis of the subclass of benign SGT trans-differentiating toward malignancy. The utilization of the quickscore takes about 15 min per case, and appears of potential great utility for routine use for pathologists. Moreover, the appreciable immunostaining for the protein on FNAB samples, gives an additional chance of a preoperative 'biological' screening of patients with PA.

For therapeutic purposes, it has to be remembered that, up to date, neither conventional chemotherapy, nor the existing targeted drugs have shown satisfactory results against these tumours (49). The ultimate significance of our results in biological terms still needs to be definitely understood. Nevertheless, they encourage us to hypothesize a role for CAF-1/p60 as a promising molecular target for therapies tailored to reverse this epigenetic alteration in aggressive SGT.

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