

HEAD AND NECK

Overexpression of chromatin assembly factor-1/p60 predicts biological behaviour of laryngeal carcinomas

L'espressività della proteina CAF-1 p60 come fattore prognostico nei carcinomi laringei

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SUMMARY

This study analysed the immunohistochemical expression of the CAF-1/p60 protein in laryngeal cancers. CAF-1/p60 assumes an independent discriminative and prognostic value in laryngeal neoplasms; the presence of this protein in carcinoma in situ compared with laryngeal precancerous and larynx infiltrating tumours. We assessed the immunohistochemical expression of CAF-1/p60 in 30 cases of moderate and/or severe dysplasia, 30 cases of carcinoma in situ and 30 cases of laryngeal squamous cell carcinoma (LSCCs). CAF-1/p60 expression increased significantly according to the high index of neoplastic cellular replication; therefore, CAF-1/p60 was overexpressed in neoplastic cells and its moderate-severe expression is correlated with poorer prognosis compared to less expression. In conclusion, overexpression of the CAF-1/p60 protein is related to a risk of higher morbidity and mortality and is a reliable independent prognostic index of laryngeal carcinoma. CAF-1-p60 protein overexpression can be used in cancer management as an indicator of malignant evolution, especially in carcinoma in situ.

KEY WORDS: CAF-1/p60 • Prognostic factor • Laryngeal cancer • Carcinoma in situ • Dysplasia • Tumoural marker

RIASSUNTO

CAF-1 è una proteina istonica trimerica implicata sia nella replicazione che nella riparazione del DNA, con il compito essenziale di stabilizzare la cromatina durante la replicazione; presenta un'azione di assemblaggio del tutto tipica, poiché unisce solo DNA che è andato incontro a replicazione. Di recente la proteina CAF-1/p60 è stata proposta come marker della proliferazione cellulare nei tumori solidi, in particolare nel distretto testa- collo in virtù della sua iper-espressione nelle cellule in stato di proliferazione rispetto a quelle quiescenti in cui è down- regolata. Questa relazione con l'attività mitotica ha inoltre permesso di considerarla come possibile indice prognostico di aggressività neoplastica. In precedenti lavori, effettuati presso il nostro dipartimento, abbiamo documentato che tale proteina risulta essere iper-espressa nei tumori del cavo orale, delle ghiandole salivari e della tiroide. In questo studio abbiamo analizzato e confrontato l'espressività immunostochimica della proteina CAF-1/p60 nelle neoformazioni laringee precancerose, nei carcinomi in situ e nei tumori maligni, in particolare: in 30 casi di displasia moderata e/o severa, 30 casi di carcinoma in situ e 30 casi di SCCs. CAF-1/p60 è iperespressa nelle cellule neoplastiche; l'espressione di CAF-1/p60 aumenta significativamente in correlazione all'alto indice di replicazione cellulare; inoltre la sua iperespressione moderata-severa è correlata con una prognosi peggiore comparata con la lieve e l'iper-espressione della proteina CAF-1/p60 è correlata con un più alto rischio di morbilità e mortalità rappresentando un reale indice prognostico delle neoplasie laringee. CAF-1/p60 assume, pertanto, un valore prognostico indipendente nelle neoplasie laringee. Riteniamo, dunque, che l'iper-espressione della proteina CAF-1/p60 che possa essere usata come indicatore di aggressività nell'evoluzione maligna specie dei carcinomi in situ ed impiegata nel follow-up per identificare le forme a più alto rischio prognostico che necessitano quindi controlli più ravvicinati nel tempo.

PAROLE CHIAVE: Proteina CAF-1/p60 • Fattori prognostici • Carcinoma della laringe • Carcinoma in situ • Displasie laringee • Lesioni precancerose • Marker tumorali

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Introduction

Laryngeal carcinoma (LC) represents one of the most common head and neck cancers and accounts for approximately 5.1% of all tumours in more developed areas and 3.5% in less developed areas, worldwide^{1,2}. The estimated incidence of laryngeal cancer in 2012 is about 1.1% of all cancers, with nearly 157, 000 new cases in 2012³.

Currently, contrary to other anatomic districts (breast, colon etc.), for LC there are no specific and sensitive markers that can be used for early diagnosis and follow-up, beyond the available prognostic parameters^{4,5}. Cell proliferative activity has been extensively investigated in head and neck tumours, including LC, as useful diagnostic and prognostic marker; however, its specific role has yet to be definitively established.

Over the last decade, it has been demonstrated that in head and neck carcinogenesis, the transition from normal epithelium to pre-malignancy, and finally to carcinoma is related to the accumulation of genetic and epigenetic alterations, is a multistep process. The most important epigenetic alterations include DNA methylation, histone modifications and RNA-mediated silencing. Chromatin assembly and remodelling is strictly regulated by histone chaperones. Chromatin assembly factor-1 (CAF-1), a histone chaperone, is an heterotrimeric protein complex formed of three subunits (p48, p60 and p150)^{6,7}. CAF-1/p60 has recently been proposed as a new sensible proliferation marker in malignant tumours^{8,9}. In particular, CAF-1/p60 is down regulated in quiescent cells, whereas it is overexpressed in hyper-proliferating and neoplastic cells¹⁰.

CAF-1 plays a crucial role in the assembly and repositioning of nucleosomes on newly synthesised DNA, regulating replication processes and DNA repair¹⁰⁻¹⁴. The p150 subunit appears to be more active in repair processes, while CAF-1/p60 is more specifically connected to controlling cell replication. The p48 subunit intervenes on acetylation/deacetylation of histones by specific protein complexes¹⁵⁻¹⁷.

In detail, CAF-1 mediates the epigenetic regulation of the state of chromatin aggregation, contributing to maintenance of chromosome structure before and after the formation of hairpin replication and appears to be involved in the transient destabilisation of nucleosomes required for the progression of hairpin replication.

CAF-1 has a typical assembly action, since it assembles only replication DNA. This is due to the fact that its activity necessarily requires interaction with the proliferating cell nuclear antigen (PCNA), which, as is known, specifically marks the newly synthesised DNA and is involved in the processes of replication, repair, recombination, repair of single strand breaks (SSB) and sister chromatid cohesion¹⁸⁻²². Ultimately, CAF-1 plays a critical role in maintaining the stability of chromatin during DNA replication and deregulation of this control mechanism can cause an uncontrolled proliferation, resulting in cancer^{10,17,18}.

In this study, we investigated the expression of CAF-1/p60 in laryngeal carcinomas to determine whether this protein could represent a reliable biological marker in evaluating tumour behaviour. Finally, we explored the possibility that this protein may represent a promising novel chance to plan for more concise follow-up, type of re-operation, or alternative treatment.

Materials and methods

Patients

The study population was selected from patients treated at the Otolaryngology, Head and Neck Surgery Department of the Federico II University, Naples, Italy, from 1 January 2000 to 31 December 2014. Inclusion criteria were:

- patients with laryngeal dysplastic precancerous lesions and laryngeal carcinoma in situ, treated surgically by excision biopsy or cordectomy (Type I, II, III, IV and V according to the classification of the European Laryngological Society, 2000) with the aid of CO₂ laser in accordance with the World Health Organization classification system (WHO) 2005²³;
- patients with early laryngeal infiltrating neoplasm (squamous cell carcinoma) treated surgically with supraglottic, subtotal or total laryngectomy;
- post-operative patients during oncological follow-up in a specialised laboratory at our clinic, with ENT specialist examinations by direct fibre optic laryngoscopy through video recording and in accordance with the timetable guidelines for each dysplastic lesion or tumour stage;
- the same selected series of patients had been evaluated for CAF-1/p60 by immunohistochemistry (Pathology section of the Department of Advanced Biomedical Sciences, Federico II University of Naples).

According to these inclusion criteria, the study population consists of 90 cases of laryngeal lesions. We divided this population into three subgroups:

- a. moderate and/ or severe dysplasia: 30 cases;
- b. carcinoma in situ: 30 cases;
- c. infiltrating cancer: 30 cases.

The population of subgroup B is composed of all consecutive cases with definitive diagnosis of carcinoma in situ (diagnosis relatively less frequent) from 1 January 2000 to 31 December 2014. To highlight the features of CAF-1/p60 expression in carcinoma in situ, this subgroup was compared to the same number of consecutive cases of dysplasia (subgroup A) and infiltrating carcinomas (subgroup C) of the larynx, from a larger series, and that respected the following inclusion criteria:

- smoking more than 20 cigarettes per day for over 20 years and who continue to smoke upon diagnosis;
- negative anamnesis for alcohol abuse;
- negative anamnesis for exposure to environmental risk factors in the work place;
- absence of any clinical and anamnestic indirect signs of gastroesophageal reflux.

Formalin-fixed paraffin-embedded blocks were selected from the archive of the Department of Advanced Biomedical Sciences, Pathology Section, Federico II University II of Naples.

For each case, paraffin blocks containing tumour areas representative of the lesion were stained for CAF-1/p60 immunohistochemical expression by comparing A, B and C subgroups.

The study was performed in accordance with the guidelines of the Institutional Ethics Committee, Italian law, and the Declaration of Helsinki, as required for studies based on retrospective analyses on routine archival formalin-fixed, paraffin-embedded tissue. All patients provided written informed consent regarding use of data.

Immunohistochemistry

For each case, 4- μ m-thick serial sections were cut and mounted on poly-L-lysine coated glass slides. Deparaffinised sections of all cases were boiled three times for 3 min in 1 mM sodium citrate buffer (pH 6.0) for antigen retrieval. In order to prevent the non-specific binding of the antibody, sections were pre-incubated with non-immune mouse serum (1:20, Dakopatts, Hamburg, Germany) diluted in PBS/BSA, 1%, for 25 min, at room temperature. After quenching of endogenous peroxidase activity was blocked by the incubation in 3% hydrogen peroxide for 30 min., followed by two rinses with Tris-HCl buffer, sections were incubated overnight at 4°C with the anti-CAF-1/p60 antibody (SS53 - ab8133, Abcam, Cambridge, MA, USA), diluted 1:300.

The standard streptavidin-biotin-peroxidase complex technique was performed, using sequential 30-min incubation with biotin-labelled secondary antibody and with peroxidase-labelled streptavidin for 30 min (DAKO LSAB kit HRP, Carpinteria, CA). For development of peroxidase activity, 3,3'-diaminobenzidine (DAB, Vector Laboratories, Burlingame, USA) was used as a substrate chromogen solution. Haematoxylin was used for nuclear counterstaining; sections were then mounted and cover-slipped with a synthetic mounting medium (Entellan, Merck, Germany). For each staining, sections from breast cancer were used as positive controls and for negative controls the sections were incubated with pre-immune serum instead of primary antibody. Only cells with definite brown nuclear staining were judged positive.

The expression of CAF-1/p60 was then rated semi-quantitatively according to an arbitrary scale, as follows: 0 (< 10% of positive cells); + (10% - < 20%); ++ (20% - < 30%); +++ (> 30% of positive cells)²⁴.

Statistical analysis

Statistical analysis was performed using Med-Calc (version 9.3.7.0), comparing the expressiveness of CAF-1/p60 between the A-B-C groups using the Wilcoxon/Mann-Whitney test for independent and non-parametric variables. According to literature, we assigned numeric values between 0 and 3 to different degrees of CAF-1/p60 expression (negative: 0; mild expression +: 1; moderate expressivity ++: 2; severe expressivity +++: 3). We applied the Kaplan-Meier method, normalising the different categories by the log-rank Mantel-Haenszel test to compare overall and specific disease survival. A Cox proportional hazard model was used to assess the simultaneous contribution of multiple factors to the risk mortality. We performed multivariate Cox regression analysis for significant variables found in univariate analyses (over-expression, staging, grading, treatment strategy, progress and distant metastasis) to underline HR of patients with moderate-severe and severe CAF-1/p60 expression. In

each test, a p value < 0.05 was considered statistically significant.

Results

The study population consists of 90 patients, 85 men and 5 women. The median age was 71 years (range, 37-86 years). Follow-up was performed in all patients (median: 33 months; range: 9-104 months).

Dysplasia

Patients with moderate and/or severe dysplasia showed a mild (+) expression of CAF-1/p60 in all cases evaluated (100%) (Fig. 1).

All patients underwent regular oncological follow-up at our clinic. During follow-up, direct laryngoscopy in 2 patients highlighted an area of suspected recurrence. These patients underwent a surgical procedure for enlargement of the previous excision (cordectomy type II vs. cordectomy type I). The histological examination relating to this procedure indicated mild dysplasia. Survival with organ and function preservation in such cases is 100% (Table I).

Carcinoma in situ

All malignant tumours showed a CAF-1/p60 overexpression (19 cases +, one case ++, 4 cases ++/ +++, 6 cases +++) (Fig. 1). During follow-up, 2 patients (6.6 %) died. The first of laryngeal carcinoma in situ (mild CAF-1/p60 expression) showed a metachronous oesophageal cancer and died of disease. In the second case, characterised by strong expression (+++) of CAF-1/p60, the death was due to recurrence and progression of disease. Twenty-eight patients (93.4%) continued oncological follow-up;

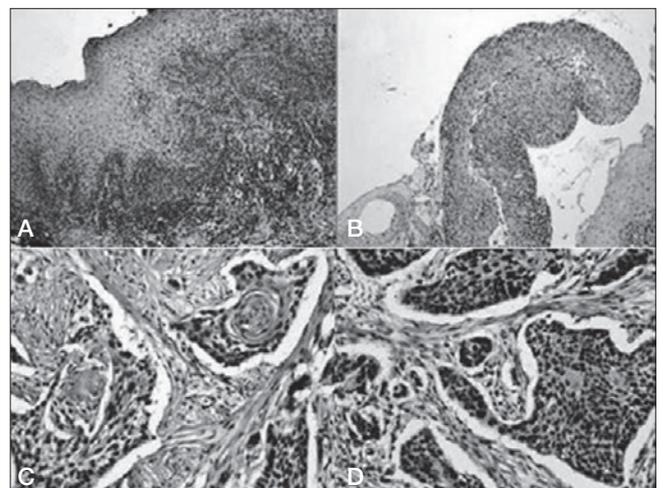


Fig. 1. A) Laryngeal mucosa with moderate-severe dysplasia and with mild (+) expression of CAF-1/p60; B) Laryngeal mucosa with cancer in situ and with severe (+++) expression of CAF-1/p60; C- D) High expression (+++) of CAF-1/p60 in moderately differentiated cancer (G2) and in undifferentiated cancer (G3).

Table I. Dysplasia; correlation of CAF-1/p60 expression with surgical treatment and follow-up.

Case	Histological diagnosis	CAF1/P60	First treatment	F-UP	Second treatment	F-UP
18	Dysplasia	+	Cordectomy type I	Normal	-	-
10	Dysplasia	+	Cordectomy type II	Normal	-	-
2	Dysplasia	+	Cordectomy type I	Relapse	Cordectomy type II	Normal

in 19 patients, the follow-up was regular without the need for further treatment (CAF-1/p60 score was + in 17 of 19 cases and ++ and ++/+++ in 2 cases). Of the remaining 9 patients whose direct laryngoscopy revealed an area of suspected relapse, 2 (CAF-1/p60 score +++) underwent excisional biopsy, with histological examination revealing varying degrees of dysplasia, while the remaining 5 (CAF-1/p60 score, respectively, one case +, one case ++/+++), 3 cases +++) underwent a second surgical procedure to enlarge the previous excision because of the location of the lesion (anterior) and positive involvement of the resection margins. Histology revealed a recurrent carcinoma in situ. Only 2 cases (CAF-1/p60 score ++/+++) underwent laryngectomy (total and supraglottic) for laryngeal disease progression. Specific survival with organ and function preservation in such cases was 96.6% considering that one patient did not die of laryngeal disease (Table II).

Infiltrating carcinoma

Patients with invasive squamous cell carcinoma were treated surgically by laryngectomy. In 18 patients (60%) the tumour was glottic, in 9 patients (30%) supraglottic and 3 patients (10%) had subglottic extension. The grading of the neoplasm was moderately differentiated (G2) in 10 patients (33.3%), from moderately to poorly differentiated (G2-G3) in 9 patients (30 %) and undifferentiated (G3) in 11 patients (36.6%).

Among the cancers with an intermediate degree of differentiation (G2), 6 of 10 cases showed a moderate expression of CAF-1/p60 (++) and continued with regular follow-up; one patient died of disease (++)), one case (++/+++) died of disease. The remaining 2 cases who had metastases at follow-up and died of the disease, showed a score of +++. All moderately to poorly differentiated (G2/G3) squamous cell carcinomas showed CAF-1/p60 moderate (++) staining in 3 cases, moderate-high (++/+++)

Table II. Cancer in situ; correlation of CAF-1/p60 expression with surgical treatment and follow-up.

Cases	Histological diagnosis	CAF-1/P60	First treatment	Follow-up	Second treatment	Follow-up
3	Ca in situ	+	Cordectomy type I	Normal	-	-
5	Ca in situ	+	Cordectomy type II	Normal	-	-
4	Ca in situ	+	Cordectomy type III	Normal	-	-
4	Ca in situ	+	Cordectomy Type IV	Normal	-	-
1	Ca in situ	+	Cordectomy type Va	Normal	-	-
1	Ca in situ	+	Cordectomy type II	Relapse ca in situ	Cordectomy type IV	Normal
1	Ca in situ	+	Cordectomy type IV	† for esophagus metachronous cancer	-	-
1	Ca in situ	++	Cordectomy type III	Normal	-	-
1	Ca in situ	++/+++	Cordectomy type III	Normal	-	-
1	Ca in situ	++/+++	Cordectomy type III	Progression of disease	TL	Normal
1	Ca in situ	++/+++	Cordectomy type IV	Relapse ca in situ	Cordectomy type Va	Normal
1	Ca in situ	++/+++	Cordectomy type Vd	Progression of disease	SGPL	Normal
1	Ca in situ	+++	Cordectomy type III	Suspect of relapse	Excisional biopsy (grave dysplasia)	4 biopsy (light dysplasia)
1	Ca in situ	+++	Cordectomy type IV	Relapse ca in situ	Cordectomy type Va	Normal
2	Ca in situ	+++	Cordectomy type Va	Relapse ca in situ	Cordectomy type Vd	Normal
1	Ca in situ	+++	Cordectomy type Va	Suspect of relapse	Excisional biopsy (light dysplasia)	Normal
1	Ca in situ	+++	Cordectomy type Vd	† for progression of disease	-	-

† death

TL = total laryngectomy

SGPL = Supraglottic partial laryngectomy

3 cases and high (+++) in the remaining 3 cases. Three cases developed metastases during follow-up (2+++ and 1 ++/+++). In poorly-differentiated squamous cell carcinomas (G3), the CAF-1/p60 was expressed at moderate levels (score ++) in 5 cases, and high levels (+++) in 6 cases; 3 patients (2+++ and 1 ++) presented metastases during follow-up (Figure 1).

Post-operative patients received oncology counselling and radiotherapy and 22 patients (73.3%) received adjuvant chemotherapy and/or radiotherapy. During follow-up at our dedicated clinic, 10 patients (33.3 %) died of disease (CAF-1/p60 score ++ in 3 cases, ++/+++ in one case and +++ in 6 cases), 8 of whom presented distant metastasis (CAF-1/p60 score +++ in 6 cases, , ++/+++ in one case and ++ in one case). Five patients died of other diseases (CAF-1/p60 score ++ in 2 cases, ++/+++ in 2 cases and +++ in one case) and the remaining 15 patients had follow-up free of disease. Survival in such cases was 66.6% considering that five patients did not die of laryngeal disease (Table III).

Statistical analysis

Statistical analysis for non-parametric variables showed a $p = 0.0015$ in comparing group A, dysplasia, with group B, carcinomas in situ of the larynx (statistically significant). Comparison between group A, dysplasia, and group C, infiltrating tumours, showed a $p < 0.0001$ (highly statistically significant). Comparison between group B, carcinoma in situ, and group C, infiltrating tumours, revealed a $p = 0.0008$ (statistically significant). Analysis of the survival rate in the study population showed a statistically significant p value ($p = 0.0178$), which became more significant in specific disease survival ($p = 0.0070$) with a cumulative survival probability of 100% within 9 months of diagnosis in patients with severe expressiveness of the CAF-1/p60 protein (+++); 100% within 16 months of diagnosis in patients with moderate-severe expressiveness of CAF-1/p60 (++/+++); 100% within 32 months of diagnosis in patients with moderate expressiveness (++) of the p60 protein. No patient with mild expressiveness died of laryngeal cancer. We deduce that the probability of an adverse event (metastasis-death) is greater in cases with severe expression (score +++) of the p60 protein (Fig. 2). A Cox proportional hazards model was used to verify whether the overexpression of the p60 protein and other variables are independent prognostic factors for LSCC patients. Univariate analysis showed that overexpression, staging, grading, treatment strategy, progress and distant metastasis were associated with survival in patients with LSCC. Multivariate analysis of the same variables showed that those variables were independent prognostic factors for patients with LSCC, and overexpression of the p60 protein was significantly associated with poor prognosis.

Table III. Infiltrating cancer; correlation of CAF-1/p60 expression with surgical treatment and follow-up.

Case	Histological diagnosis	Grading/staging	CAF-1/P60	Surgical treatment	F-UP
1	LSCC	II/ G2	+++	SCPL	† - M+
2	LSCC	II/ G2	++	TL	Normal
3	LSCC	III/ G2-G3	++	SCPL	Normal
4	LSCC	III/ G2- G3	++	SGPL	†
5	LSCC	IVa/ G2	+++	SCPL	† - M+
6	LSCC	IVa/ G2	++	SCPL	Normal
7	LSCC	IVa/ G2- G3	++	SCPL	Normal
8	LSCC	IVa/ G3	++	SGPL	† for other disease
9	LSCC	IVa/ G3	++	SGPL	Normal
10	LSCC	IVa/ G3	++	SCPL	† - M+
11	LSCC	IVa/ G3	++	TL	† for other disease
12	LSCC	II/G2	++/+++	SCPL	† for other disease
13	LSCC	IVa/G2- G3	++/+++	SCPL	† for other disease
14	LSCC	II/ G2	++	SGPL	Normal
15	LSCC	III/ G3	++	SCPL	Normal
16	LSCC	IVa/ G2- G3	+++	SGPL	† - M+
17	LSCC	III/ G2	++	SGPL	Normal
18	LSCC	IVa/ G3	+++	SCPL	† - M+
19	LSCC	IVa/ G3	+++	TL	† - M+
20	LSCC	IVa/ G2	++	SGPL	Normal
21	LSCC	IVa/ G3	+++	SCPL	Normal
22	LSCC	III/ G2- G3	+++	SGPL	† for other disease
23	LSCC	IVa/ G2	++	SCPL	†
24	LSCC	IVa/ G3	+++	SCPL	Normal
25	LSCC	IVa/ G3	+++	TL	Normal
26	LSCC	IVa/ G2- G3	++/ +++	SCPL	† - M+
27	LSCC	II/ G2	++	SCPL	Normal
28	LSCC	IVa/ G3	+++	SCPL	Normal
29	LSCC	IVa/ G2- G3	++/+++	SCPL	Normal
30	LSCC	IVa/ G2- G3	+++	TL	† - M+

Abbreviations

LSCC: squamous cell carcinomas of the larynx; SCPL: supracricoid partial laryngectomy; SGPL: supraglottic partial laryngectomy; TL: total laryngectomy; † death; M+ metastasis

Discussion

Knowledge of the multistep laryngeal carcinogenesis mechanism is a prerequisite for the development of cancer prevention and treatment strategies. CAF1/p60 has been proposed as a new proliferation and prognostic marker in a series of different malignant tumours. The expression of the p60 subunit is particularly high in neoplasms with increased cellular proliferation: while CAF-1/p60 is down-regulated in resting cells, it is greatly overexpressed in neoplastic cells⁸. Moreover, the dissociation of CAF-1/p60 from hair-

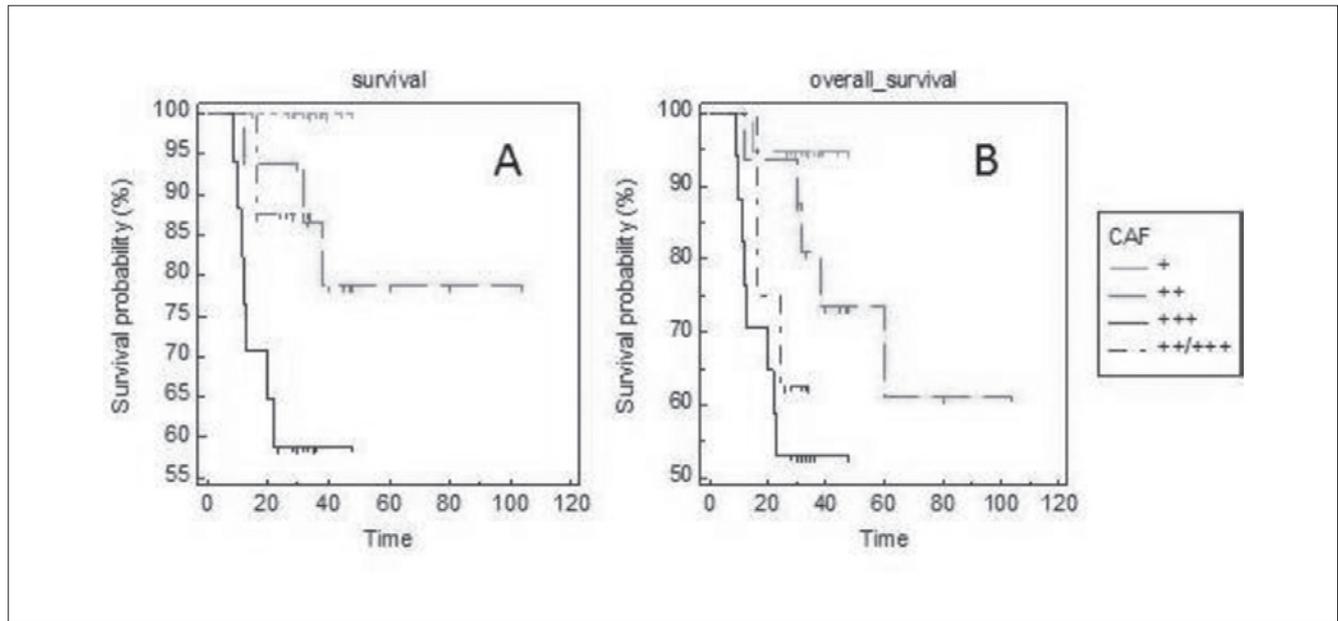


Fig. 2. Analysis of survival; A) disease-specific survival and B) overall survival.

pin replication causes the suspension of its activity in the nucleosomes assembly, with the arrest of hairpin replication, mitosis blocking during the cellular cycle and global alterations of chromatin in the S phase²⁵. In addition to being a reliable marker of cellular proliferation, CAF-1/p60 also assumes a relevant value as reliable marker of tumour progression and as a predictor of biological behaviour^{26,27}.

In fact, several recent reports have highlighted the strict relationship between CAF-1/p60 over-expression and adverse behaviour of different malignant tumours, including prostate, tongue, salivary glands carcinomas and cutaneous melanoma²⁸⁻³¹. These studies have confirmed the hypothesis that CAF-1/p60 is heavily involved in tumoural and metastatic processes, constituting an independent prognostic biomarker of tumour evolution^{8,25,32-34}.

Our results indicate that the levels of expression of the CAF-1/p60 assume a significant ($p < 0.05$) value for moderate/severe dysplastic lesions of the larynx compared with carcinomas in situ and when comparing both dysplasia and carcinomas in situ with infiltrating carcinomas of the larynx. Analysis of the surgical procedure allows us to confirm that excisional biopsy is a valid tool in treating the dysplasia, decisive in 100% of cases, while for carcinomas in situ excisional biopsy was the sole treatment performed in 19 patients, while a second surgical treatment was required in 9 patients. CAF-1/p60 expression was mild in all evaluated epithelial laryngeal dysplasia (100%). With reference to the clinical behaviour of carcinoma in situ, we can assume that mild overexpression of CAF-1/p60 (mild +) is connected to a regular progress; in patients with a moderate to severe expression on the other hand there is an increase in repeat surgery and relapses.

It is possible to speculate that in cases of carcinoma in situ in which p60 is overexpressed, close follow-up is required to assess possible recurrence as promptly as possible and plan appropriate treatment. In infiltrating tumours of the larynx, p60 protein expression is moderate to high. In these patients, there is an increase in mortality and recurrence in accordance with the standard parameters of neoplasia (staging, grading); however, overexpression of CAF-1/p60 appears to be related to an independent risk of mortality associated with distant metastases, which are not common in tumours of the larynx. In fact, in 8 patients with moderate to high expression, the course of the disease was ominous, with distant metastases leading

Covariates	b	SE	P	HR Exp(b)	95% CI of Exp(b)	p
Overexpression	0,948 4	0,325 4	0,00366 9	2,5815	1,3659 to 4,8788	P = 0,0009
Staging	0,804 5	0,297 9	0,00691 2	2,2357	1,2507 to 3,9963	P = 0,0013
Grading	0,595 0	0,249 5	0,01711 3	1,8130	1,1145 to 2,9495	P = 0,0155
Treatment	1,072 9	0,330 1	0,00115 3	2,9259	1,5361 to 5,5655	P = 0,0001
Progress	2,864 1	0,881 3	0,00115 5	17,5332	3,1441 to 97,7750	P < 0,0001
Distant metastasis	2,881 6	0,843 0	0,00146 8	14,6078	2,8228 to 75,5952	P < 0,0001

Fig. 3. Univariate analyses of overexpression, staging, treatment strategy, progression and distant metastases.

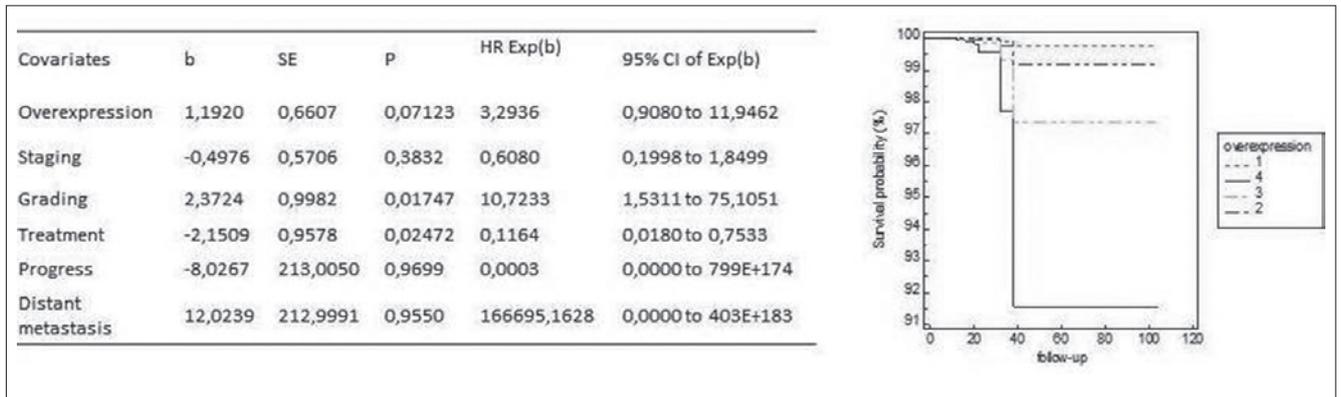


Fig. 4. Multivariate Cox regression analysis.

to death of both patients. This suggests that the overexpression of CAF can identify the subset of tumours with a more aggressive biological from among those having equivalent staging and grading.

In 36.6% of patients (11 cases) with carcinoma in situ, immunopositivity was moderate to high. In 9 patients, further surgery was required (5 cases of recurrence of carcinoma in situ, one case of laryngectomy and one patient death due to recurrence and progression of laryngeal cancer). The highest expression of protein characterised cases of metastatic SCC and the majority of carcinoma in situ (all except one) with a history of progressive disease (recurrence and/ or death due to disease). This clinical behaviour (need for further surgery, relapse and progression) highlights the need for a tighter follow-up timetable as a marker for such carcinoma in situ patients.

Analyses of survival indicate an increased risk of recurrence and mortality concurrent with the parameters assessed by univariate analyses that assume a predictive and statistical significance in each case (Fig. 3). Multivariate analysis of these variables showed that staging, grading, treatment strategy, progress, distant metastasis are all independent prognostic factors for patients with LSCCs, and overexpression of CAF-1/p60 protein is significantly associated with poor prognosis.

Multivariate analysis correlating risk of overexpression, considering the prognostic factors examined in the univariate analyse shows an increase in HR (2.58 versus 3.29) compared to univariate analysis with a $p < 0.001$ (Fig. 4). Also worthy of note is the HR of the distant metastases, an expression of the aggressiveness of the disease.

Conclusions

Our results show that CAF-1/p60 in an independent prognostic factor that may better predict the biological behaviour of LSCC associated with traditional prognostic features, but this exciting hypothesis needs to be validated by a larger and more representative series of cases. If confirmed, we may adopt a more pronounced oncological follow-up protocol for the subset of patients with carcinoma

in situ overexpressing CAF-1/p60 and adjuvant therapy programmes in infiltrating ones.

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