Expression of the Apoptosis Inhibitor Protein *Survivin* in Primary Laryngeal Carcinoma and Cervical Lymph Node Metastasis

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Abstract. *Background: Survivin is the smallest mammalian member of the inhibitor of apoptosis (IAP) proteins family. The aim of the present study was to determine survivin expression in laryngeal squamous cell carcinoma (SCC) and in neck lymph node metastases. The survivin value in predicting prognosis in laryngeal SCC was also investigated. Patients and Methods: Survivin expression was investigated in 37 laryngeal SCCs and in 12 cervical lymph node metastases. Results: A nuclear reaction predominated in laryngeal SCCs. Survivin expression was significantly higher in pN+ laryngeal SCCs than in pN0 (p=0.017). Survivin expression was higher in cervical lymph node metastases than in correspondent primary laryngeal SCCs (p=0.002). Survivin expression in laryngeal SCCs that developed loco-regional recurrence was significantly higher than in laryngeal SCCs without recurrence of disease (p=0.039). Conclusion: Nuclear expression of survivin should be studied as a hallmark of higher risk laryngeal SCCs to develop loco-regional recurrences. Higher survivin expression in pN+ laryngeal SCC may suggest elective neck dissection in clinically N0 patients with high survivin expression in primary SCC. The extremely high survivin expression in lymph node metastases may support the use of survivin in the diagnosis of lymph node micro-metastases.*

Apoptosis is an active mechanism leading to cell death, which controls the development and homeostasis of multicellular organisms. Tight regulation is required to ensure a delicate balance of cell life and death. Indeed, loss of apoptotic regulation results in a wide variety of diseases. Cellular defects that halt apoptosis are frequently involved in cancer development and progression.

Apoptosis inhibitor proteins (IAPs) are a group of structurally related, anti-apoptotic proteins. The IAPs are over-expressed in many cases of human malignant tissues. *Survivin* is the smallest mammalian member of the IAP gene family. A single-copy *survivin* gene is located on human chromosome 17q25. The *survivin* gene is expressed as a 16.5-kDa protein. Significant over-expression of *survivin* has been demonstrated in tumours of lung, breast, colon, stomach, oesophagus, pancreas, liver, uterus, ovaries, Hodgkin's disease, non-Hodgkin's lymphoma, leukemias, myelodysplastic syndrome with refractory anaemia, neuroblastoma, pheochromocytoma, soft tissue sarcomas, brain tumours and melanoma (1-3). *Survivin* expression in oral (4-7), oral and oropharyngeal (8), tonsillar (9) and nasopharyngeal (10) squamous cell carcinomas (SCCs) have also been investigated.

The aim of the present study was to determine the expression of the IAP *survivin* in laryngeal SCCs and in their neck lymph node metastases. The value of *survivin* in predicting prognosis in a cohort of patients with laryngeal SCC treated with partial laryngectomy, with or without neck dissection, was investigated.

Patients and Methods

Patients. A total of 37 cases of laryngeal SCC were evaluated. Thirty-four cases were male and 3 cases female, with a mean age of 63 years (range 43-73 years). All patients underwent partial laryngectomy at the Department of Otolaryngology, Head and Neck Surgery of Padova University, Italy. In 33 out of 37 cases (89.2%) neck dissection was performed. Five patients underwent also post-operative radiotherapy. According to TNM Classification of Malignant Tumours of the International Union Against Cancer

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(11), the pathological staging of primary laryngeal lesions (pT) was T1 in 6 cases, T2 in 18 cases, T3 in 11 and T4 in 2. Pathologic regional lymph node staging was N0 in 21 cases, N1 in 8 cases, N2b in 2 cases and N2c in 2 cases. According to histopathological grading, 10 laryngeal SCCs were staged G1, 16 G2, 11 G3. The mean follow-up time was 39.3 months (median 42 months, standard deviation (SD) 17.5 months).

All tissues were fixed in 10% formalin and embedded in paraffin wax.

Immunohistochemistry. From each of the 49 tissue blocks (37 primary laryngeal SCCs, 12 cervical lymph node metastases), 5-micron sections were cut for immunohistochemistry, deparaffinised and re-hydrated. Haematoxylin-eosin staining was performed on one section from each tissue block to confirm the diagnosis of SCC. For each sample *survivin* reactivity was evaluated. The sections were pre-treated in a microwave oven (750 Watt) for 20 min in a citrate buffer (10 mM, pH 6.0). The sections were pre-incubated with super block (Ultra tech HRP) for 10 min to block non-specific background staining. The sections were then incubated with rabbit polyclonal antibody *Survivin AB-6* (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1:300 diluted for 45 min at room temperature and washed with phosphate-buffered saline (PBS) (pH 7.00) for 3 min. The second layer was incubated in biotinylated pre-diluted antibody (Ultra Tek HRP-Anti Polyvalent Scytek, Logan, UT) for 10 min. The sections were incubated with streptoavidin peroxidase complex (Ultra Tek HRP) for 10 min. Between incubations, the sections were washed in PBS (pH 7.00) for 3 min. Finally, the colour was developed using 3,3'-diaminobenzidine (DAB) (DAKO, Glostrup, Denmark) for 4 min. Sections were counterstained with Meyer haematoxylin. Negative controls in the absence of primary antibodies were also obtained.

For each case, 40 randomly non-overlapping fields of SCC were evaluated at 400X magnification by the histopathologist (F.M.). Survivin expression was measured as a percentage of *survivin*-stained carcinoma cells.

Statistical analysis. When appropriate, the following statistical tests were applied: one-way analysis of variance, Wilcoxon signed rank test (paired data). Cox proportional hazard model was applied to assess the relation between *survivin* expression and disease-free intervals (in months). A significance level of 0.05 (two-tailed) was assumed for all the calculations, to determine whether to reject the null hypothesis. *P*-values inside the range $0.10>p\geq0.05$ were considered to indicate a statistical trend.

Results

Normal epithelial cells of laryngeal mucosa showed weak immunohistochemical staining for *survivin* in sporadic groups of cells of the basal and parabasal layers (Figure 1A).

In most of the considered specimens of primary laryngeal SCC, a nuclear reaction predominated and no or only scattered cells exhibited a cytoplasmic reaction. The mean *survivin* expression in primary laryngeal SCCs was 21.5% (median 20.0%, SD 11.7%). *Survivin* expression in the laryngeal carcinomas ranged from 2% to 40%. (Figure 1B, C).

The mean *survivin* expressions were 18.8% (median 15.0%, SD 11.3%) and 28.3% (median 30.0%, SD 8.9%) in pN0 (21 cases) and pN+ (12 cases) carcinomas, respectively. One-way analysis of variance disclosed a statistical difference between these two groups ($F=6.29$, $p=0.017$).

Table I. *Survivin expression in laryngeal SCC. Most relevant results and statistical analyses.*

	No. cases	Mean *survivin* expression (SD)	Statistical analysis
pN+	12	28.3% (8.9%)	
pN0	21	18.8% (11.3%)	$p=0.017$
Primary SCCs (pN+)	12	28.3% (8.9%)	
Correspondent lymph node metastases	12	67.5% (10.6%)	$p=0.002$
Without L-R Rec	27	19.1% (11.3%)	
With L-R Rec	10	28.0% (10.9%)	$p=0.039$

L-R Rec: loco-regional recurrence; SD: standard deviation.

The mean *survivin* expressions in primary laryngeal pN+ SCCs and in their cervical lymph node metastases were 28.3% (median 30.0%, SD 8.9%) and 67.5% (median 70.0%, SD 15.5%), respectively. Nuclear *survivin* expression also predominated in lymph node metastases (Figure 1D). Wilcoxon signed rank test showed a statistically significant difference between primary lesions and corresponding neck metastases ($p=0.002$).

The mean *survivin* expression was 23.3% (median 25.0%; SD 14.0%) in pT1 carcinomas, 24.4% (median 27.5%; SD 10.9%) in pT2 carcinomas, and 17.5% (median 15.0%; SD 11.8%) in pT3. The one-way analysis of variance failed to identify any significant relationship between T stage and *survivin* expression ($F=1.28$, $p=0.29$).

The mean *survivin* expression was 21.0% (median 17.5%; SD 10.5%) in G1 carcinomas, 18.1% (median 17.5%; SD 11.1%) in G2 and 27.0% (median 30.0%; SD 12.7%) in G3. The one-way analysis of variance did not show any statistical correlation between *survivin* expression and laryngeal SCC histopathological grading ($F=1.98$, $p=0.15$).

Twenty-seven out of the 37 considered cases of laryngeal SCC did not experience malignancy recurrence. Ten patients developed loco-regional malignancy recurrence after a mean period of 18.1 months (median 11.0 months, SD 15.5 months). The mean *survivin* expression in primary laryngeal SCCs with loco-regional recurrence was 28.0% (median 30.0%; SD 10.9%). The mean *survivin* expression in laryngeal SCCs without recurrence was 19.1% (median 15.0%; SD 11.3%). The one-way analysis of variance

showed a statistically significant difference in *survivin* expression between these two prognostic groups (loco-regional recurrence *vs.* no recurrence) (F=4.56, p=0.039).

The Cox proportional hazard model showed a trend towards statistical correlation between nuclear *survivin* expression in laryngeal SCCs and disease-free intervals after treatment calculated in months (hazard ratio=1.05, standard error=0.031, p=0.069, 95% confidence interval 0.99-1.12).

The most relevant results and statistical analyses are summarised in Table I.

Discussion

Survivin expression and its prognostic role were investigated in laryngeal SCC only by few previous studies published in English (12-15). Very recently, our group (16) compared *survivin* expression in laryngeal basaloid SCCs (an uncommon bimorphic variant of SSC) and site-matched, stage-matched conventional laryngeal SCCs.

In contrast to previous studies that investigated *survivin* expression in laryngeal SCC biopsies and surgical specimens (12) or biopsies (13), only surgical specimens were considered here to avoid the selection bias related to the biopsy procedures. We evaluated *survivin* expression in surgical specimens (neck dissection) of laryngeal SCC cervical lymph node metastases.

Although several reports stated that normal mucosa specimens from superior aero-digestive tract were negative for *survivin* expression (12-14), the results of our study showed weak *survivin* staining in non-neoplastic cells of the basal and parabasal layers of laryngeal mucosa. This evidence was reported also by Lo Muzio *et al.* (17), Weinman *et al.* (9), Marioni *et al.* (8) and Marioni *et al.* (16). These results also support the recent evidence that *survivin* is expressed in normal adult tissues, not only in the thymus and testis, but also in proliferating normal adult tissues.

Subcellular localisation of *survivin* expression in malignant cells is still controversial. *Survivin* seems to exist in 2 subcellular pools (cytoplasmic and nuclear). *Survivin* splice patterns may affect the subcellular localisation, although the mechanisms controlling the splicing process remain unclear. Recently, Grabowski and coworkers (18) stated that in oesophageal carcinoma, nuclear *survivin* expression was nearly invariably associated with poor prognosis and cytoplasmic staining had no prognostic relevance. In our series of laryngeal SCCs, *survivin* nuclear reaction predominated and none or only scattered cells exhibited an exclusively cytoplasmic reaction in agreement with a study by Pizem *et al.* (13).

In our series, *survivin* expression was significantly higher in pN+ laryngeal SCCs than in pN0. This evidence was also provided by Dong *et al.* (12) in laryngeal SCCs, Lo Muzio *et al.* (4) in oral SCCs and Marioni *et al.* (8) in oral and oropharyngeal SCCs. Considering that highly metastatic cancers exhibit a higher resistance to apoptotic cell death compared to low metastatic forms, our data suggest that *survivin* expression in aggressive laryngeal SCC may provide a strong growth advantage factor for tumour progression and metastasis development. In 2006, our group investigated *survivin* expression for the first time in a very limited series of lymph node cervical metastases due to laryngeal SCC and basaloid SCC (16). The present study indicates that *survivin* expression was significantly higher in the cervical lymph node metastases than in corresponding primary laryngeal SCCs (67.5% *vs.* 28.3%; p=0.002). One possible explanation may be that *survivin* expression is up-regulated in metastatic carcinoma cells. It is known that failures in normal apoptosis pathways could contribute to carcinoma progression by supporting anchorage-independent survival during metastasis (19). Considering the anti-apoptotic function of *survivin*, it seems reasonable to hypothesise that *survivin* expression might facilitate the survival of carcinoma cells at distant sites.

Comprehensively, our data suggest that nuclear *survivin* expression heralds a poor prognosis in laryngeal SCC. In the present series, *survivin* expression in primary laryngeal SCCs that developed loco-regional recurrence was significantly higher than *survivin* expression in laryngeal SCCs without recurrence of disease after treatment (p=0.039). The data analysis also showed a trend towards statistical inverse correlation between nuclear *survivin* expression in laryngeal SCCs and disease-free intervals after treatment (p=0.069).

Conclusion

More research and improved prognostication would be clinically valuable, particularly where initial therapy may be tailored to counter variable laryngeal SCC aggressiveness. Considering the present evidence, nuclear expression of *survivin* should be studied as a promising hallmark of higher risk laryngeal SCCs to develop loco-regional recurrences.

A large series confirmation of higher *survivin* expression in laryngeal SCC with lymph node metastases may imply the necessity for elective neck dissection in clinically N0 patients with primary SCC high *survivin* expression.

The extremely high *survivin* expression rate in cervical lymph node metastases suggests a potential approach, using *survivin* as a molecular marker, for the diagnosis of lymph node micro-metastases in laryngeal SCC.

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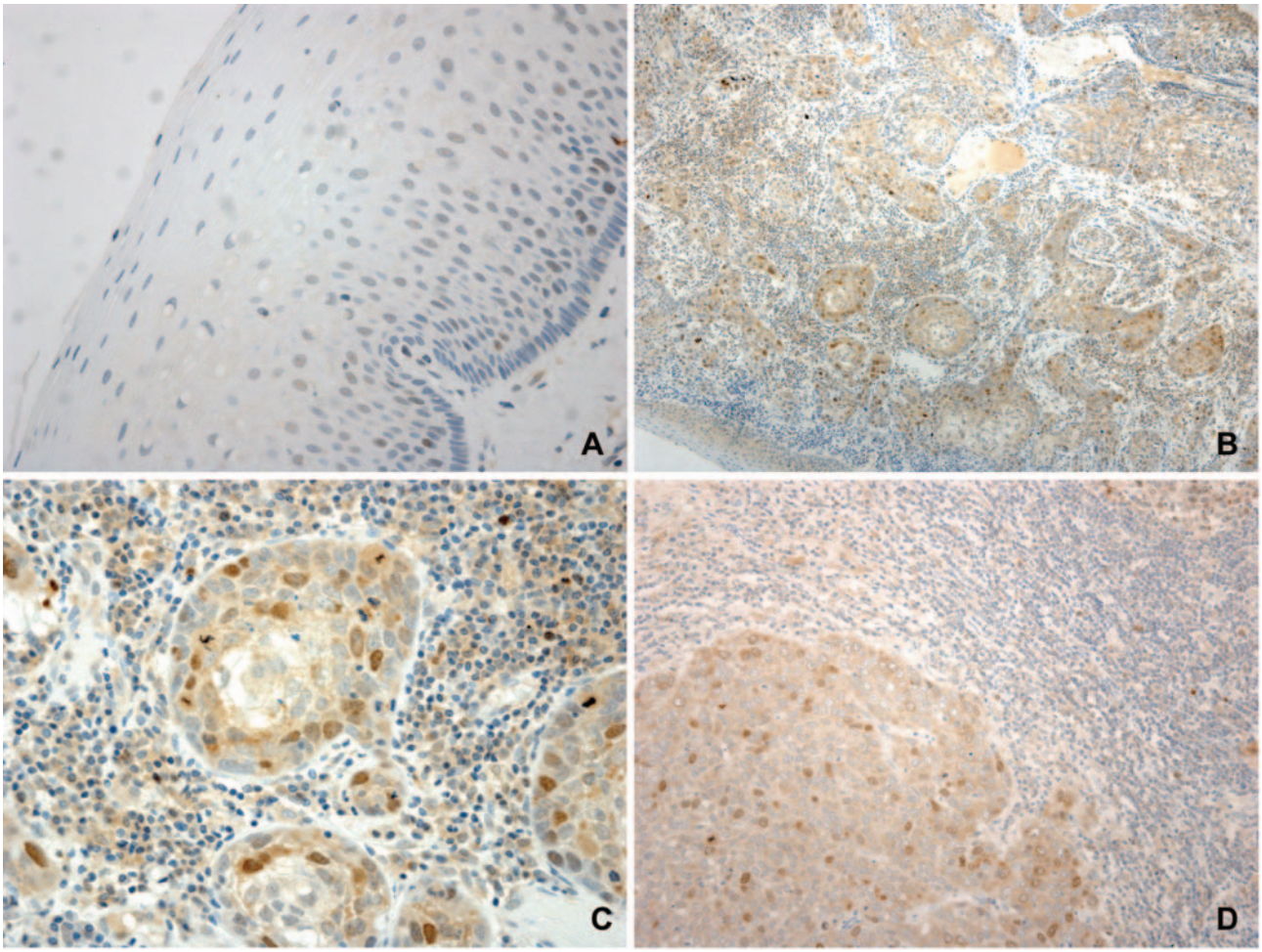

Figure 1. *A) Nuclear survivin immunoreactivity in basal cells of normal laryngeal epithelium (Survivin AB-6 [Santa Cruz Biotechnology, Santa Cruz, CA, USA]; original magnification x200); B) and C) Nuclear immunoreactivity for survivin in laryngeal squamous cell carcinoma (SCC) (original magnification x100 and x400, respectively); D) Nuclear immunoreactivity for survivin in a cervical lymph node metastasis due to laryngeal primary SCC (original magnification x400).*

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