

Immunodetection and clinico-pathological correlates of two tumour growth regulators in laryngeal carcinoma

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Summary. Activation of telomerase, present in the vast majority of all human cancers, is associated with elongation of chromosomal telomeres and consequent cell immortalization. Telomere length homeostasis is a dynamic process governed by the negative feedback mechanism of the telomeric repeat binding factor 1 (TRF1) which inhibits the action of telomerase in telomerase-positive cells. In an attempt to investigate markers of tumour growth as possible prognostic indicators in laryngeal cancer, we studied the expression of TRF1 and of the proliferation marker Ki67 on 96 invasive squamous carcinomas of the larynx. A standard three step immunoperoxidase staining method was applied on paraffin sections incubated with appropriate polyclonal antibodies. The percentages of Ki67- and TRF1-immunopositive cancerous cells were calculated by image analysis. Univariate and multivariate statistical analysis of the staining results were performed in order to detect any association of the examined immunomarkers with the tumours' classical clinicopathological variables including nuclear morphometric features as well as with patients' disease-free survival. Ki67 immunostaining was positively linked with advanced patients' age, nodal involvement as well as presence of early recurrence. No relation was found between proliferative fraction and TRF1 immunoexpression. TRF1 was expressed in 55.2% of all cases and was positively linked only to tumour size. Multivariate statistical analysis revealed the presence of lymph nodal metastasis and Ki67 immunopositivity index 20% as significant predictors of relapse. Increased Ki67 immunostaining appears to be a promising marker of tumour aggressiveness in laryngeal cancer. After one point at the tumour's natural history, the maintenance of tumour growth does not seem to depend on cell proliferation but on TRF1 immunoexpression. Whether the latter can be used for the identification of immortalized cells in every-day

practice is worth investigating.

Key words: Larynx, squamous cell carcinoma, Ki67, TRF1, Immunohistochemistry

Introduction

The prognosis of laryngeal squamous cell carcinoma (LSCC) has so far been mainly based on traditional pathological parameters (Resnick et al., 1995; Valente et al., 1999). Nevertheless, patients with similar pathological variables that have undergone the same treatment may have a different outcome; this prompted pathologists to investigate the usefulness of a number of ancillary markers in an effort to add more detailed prognostic information to the routine histopathological factors.

The proliferation of neoplastic cells may be an important prognostic indicator for a variety of different malignancies since tumour cell kinetics directly affect the clinical course of patients suffering from cancer. Among techniques that enable estimation of proliferative activity, immunohistochemical detection of proteins associated with cell proliferation has gained much attention in the past few years and antibodies to Ki67 protein have been widely applied. The latter react with the nuclear Ki67 antigen which is present in G1, S and G2 phases of the cell cycle as well as in mitosis (M) but is absent in G0 phase (Pignataro et al., 1998).

In addition to cell proliferation, tumour growth must logically depend on the proportion of "immortalized" neoplastic cells among the whole neoplastic population. Telomeres are specialized nucleoprotein complexes that form the ends of eucariotic chromosomes (König et al., 1998); because these ends cannot be replicated, each successive round of cell division causes a shortening of the telomeres eventually leading to senescence and apoptosis. However, immortal cells manage to maintain stable telomeres owing to telomerase activity. Telomerase is a ribonucleoprotein enzyme that adds telomeric repeats onto the ends of chromosomes during the replicative phase (S-phase) of the cell cycle (Zhu et

al., 1996), thus preventing cellular senescence. Telomerase is present in human embryonic tissues, is not detected in most adult tissues, but is upregulated or reactivated in almost 90% of all human cancers (Shay, 1997), probably giving tumour cells a growth advantage over normal cells (Kyo et al., 2000); actually, telomerase activity has been reported in a high proportion of oral, head and neck carcinomas (Chiu and Harley, 1997; Thurnher et al., 1998; Sumida et al., 1999; Koscielny et al., 2000) and of the larynx, in particular (Hohaus et al., 1996; Curran et al., 2000). It is being postulated that a mechanism to restore and maintain telomeres (such as activation of telomerase) is required for tumour progression and cellular immortalization (Chong et al., 1995); so, telomerase activation is expected to be negatively associated with outcome measures in patients with cancer. It is noteworthy that telomerase expression in immortalized human cells does not lead to unlimited telomere elongation. A major protein component of human telomeres (Chong et al., 1995), telomeric repeat binding factor 1 (TRF1) has been shown to stabilize telomere length in telomerase-positive cells (Shen et al., 1997) by suppressing telomere elongation (van Steensel and de Lange, 1997). TRF1 is dramatically increased in G2+M phases of the cell cycle; TRF1 presence is directly linked with the previous activity of telomerase which, as already mentioned, reaches its maximum in the S phase of the cell cycle (Zhu et al., 1996); thus, TRF1 protein could logically be used as an indirect immortalization marker of the cells in which it is expressed.

The aim of the present study was to investigate the immunohistochemical expression of the above-mentioned regulators of tumour growth (i.e., cell proliferation and immortalization) in a well documented series of LSCCs, to assess the proportion of cycling and immortalized neoplastic cells and to look for any possible associations with the tumours' clinicopathological factors including nuclear morphometric features and patients' recurrence-free survival.

Materials and methods

Ninety-six patients with conventional invasive LSCC (mean age: 62.7 yrs, SD: 8.5 yrs, range: 40 yrs) were surgically treated and followed up from 8 to 72 months (median of 40 months). A smoking history was positive in 91 patients. In all cases, despite differences in the type of laryngectomy, the surgical margins were free of tumour. Seventy-three patients had undergone either a selective or radial neck dissection. Sixty out of the 96 patients relapsed. The range of relapse time was 2-28 months (mean: 11.8). Table 1 stratifies the tumours according to their main clinicopathological features. The histological grading was evaluated following the criteria proposed by the WHO (Shanmugaratnam, 1991).

Formalin-fixed, paraffin-embedded tissue sections were stained by means of standard three-step

immunohistochemistry (ABC method) and diaminobenzidine substrate. Dewaxed sections were heated in 0.01M citric acid (pH 6.0) for two times ten minutes in a microwave oven at 700 Watts. The primary antibodies (Abs) employed were the goat polyclonal Ab C-19 (Santa Cruz Biotechnology, California, USA) and a highly specific DAKO rabbit anti-human Ki67 antigen Ab, for the detection of TRF1 and Ki67, respectively. Both antibodies were diluted 150 times and incubated overnight. The primary antibodies were omitted from negative controls. A previously TRF1-positive tissue section of LSCC was used as a positive control for TRF1 staining in each experiment.

The estimation of immunostained malignant cells as well as of nuclear morphometric characteristics of malignant nuclei (which may serve as markers of aggressive tumour biology) was performed by image analysis. Images were acquired using a Zeiss Axiolab microscope (Zeiss GmbH, Jena, Germany) with a mechanical stage, fitted with a Sony-iris CCD videocamera (SONY Corp., Tokyo, Japan). The videocamera was connected to a Pentium II personal computer loaded with the appropriate image analysis software (Image Scan, Jandel Scientific, Erkrath, Germany). Firstly, we calculated the ratio expressed in % between the number of the immunohistochemically-positive stained neoplastic cells and the total number (stained and unstained ones). Ki67 and TRF1 indices were reported as the percentages of immunoreactive malignant cells (Fig. 1). Then we measured the intensity of the immunohistochemical staining classifying all the positively-stained neoplastic cells into two groups (weakly stained and strongly stained). Staining intensity of the immunopositive cells was not associated with any of the parameters examined. The morphometric measurements were performed with the above-mentioned software. We measured four nuclear geometric parameters i.e. major axis length, minor axis length, perimeter and area. In order to determine the contours of the malignant nuclei, automatic segmentation of the boundaries of each nucleus was performed. For analysis of each image, the operator adjusted the gray level between each of the nuclei

Table 1. Clinicopathological features of the examined tumours.

A. Patient's Sex	Male (n=91) Female (n=5)
B. Tumour Location	Glottic (n=37) Transglottic (n=10) Supraglottic (n=34) Subglottic (n=15)
C. TNM Stage	I (n=30) II (n=24) III (n=28) IV (n=14)
D. Histological Grade	Well differentiated (n=28) Moderately differentiated (n=52) Poorly differentiated (n=16)

analyzed and the background. The contours of these nuclei (i.e., connected components darker than the threshold) were then captured automatically. Usually automatic segmentation was only partly successful and contours were manually edited or drawn with the mouse.

Morphometric characteristics, Ki67 and TRF1 immunopositivity per cent rates were treated as continuous variables. Data are reported as mean±SD. Variables of interest were compared with: a) stage and grade using one way analysis of variance (ANOVA); and b) with location and presence of lymph node metastasis using students' t-test (2-tailed). In addition, for statistical purposes, cases were divided into three groups with regard to Ki67 immunopositivity percentages [i.e. (group A): low Ki67 immunoreactivity (<20% of malignant cells), (group B): moderate Ki67 immunoreactivity (20-40% of cancerous cells) and (group C): high Ki67 immunopositivity (>40% of malignant cells)]. Multiple linear regression was employed to assess multiple correlations of Ki67 and TRF1 expression [three groups for Ki67 and two groups (i.e. positive vs negative) for TRF1] with age, tumour location, stage, grade, morphometric characteristics of cancerous cells and lymph nodal involvement. The hypothesis that relapse time is associated with some of the evaluated clinicopathological parameters was statistically investigated; the Kaplan-Meier method was employed to

contrast Hazard curves and log-ranks. Logistic regression model and Cox's regression model utilizing stepwise method were used to determine the effect of TRF1 and Ki67 to relapse-free survival after adjustment for the other risk factors. A value of less than 0.05 was considered significant.

Results

Ki67 immunopositivity

Ki67-positive malignant nuclei were noticeable in all examined specimens (mean±SD: 30.385±22.623%, range: 2.10-80%). In tumour-adjacent mucosa, Ki67-positive dysplastic cells were observable throughout the entire thickness of the epithelium (Fig. 2a). In well differentiated, keratinizing LSCCs, Ki67 tended to stain the basaloid tumour cells at the periphery of the tumour islands; in less differentiated, non-keratinising carcinomas, the number of Ki67-positive cells was dispersed throughout the sample (Fig. 2b). In some cases the majority of Ki67 expressing neoplastic cells was observed within the tumours' invasive margin. Increased Ki67 immunoreactivity was more frequently detected among patients of advanced age ($p<0.0005$) and with nodal metastases (multiple linear regression model: $p=0.025$, ANOVA: $p=0.028$) (Fig. 3a). No other

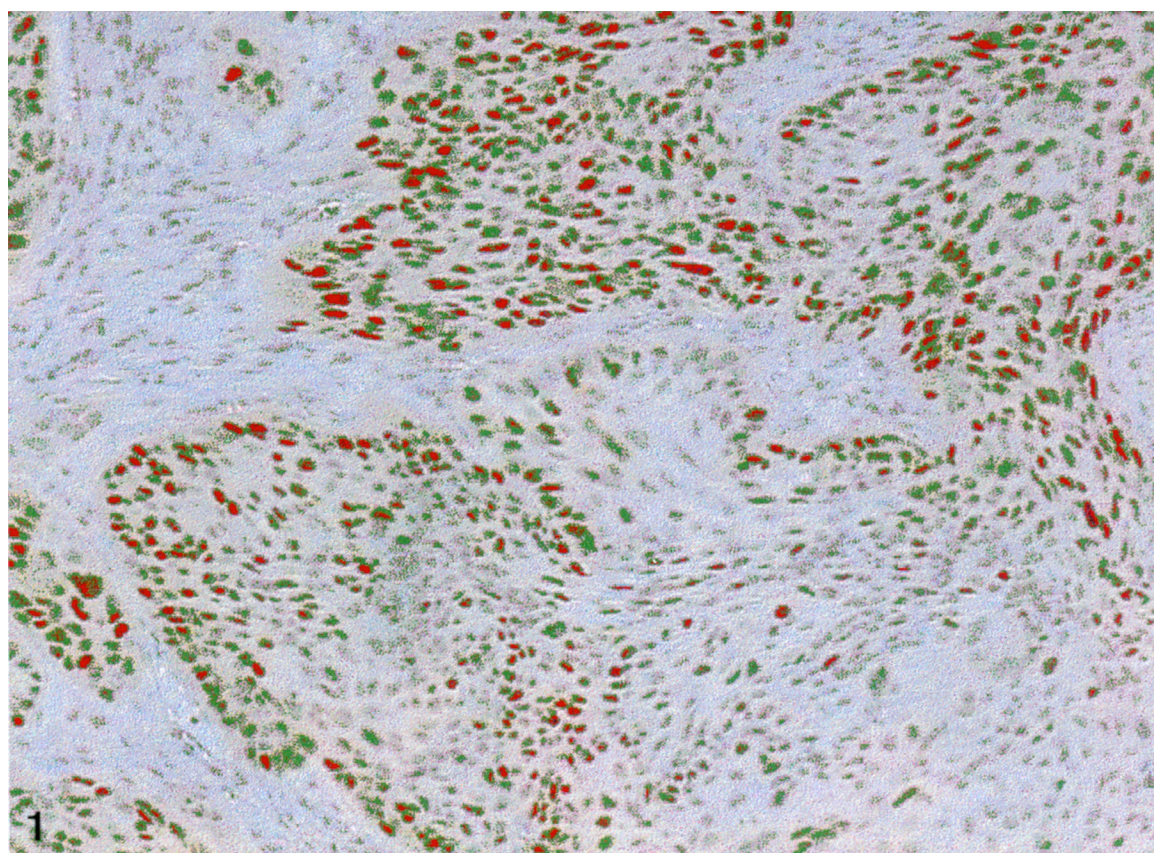


Fig. 1. Computerized image of a laryngeal squamous cell carcinoma stained for Ki67 (x 250). The image is elaborated with the appropriate morphometry software (Note the dotted nuclei).

clinicopathological or morphometric feature was associated with Ki67 immunostaining. As concerns relapse-free survival, tumours with Ki67 values lower than 20% did not relapse during the follow-up period; there was a statistically significant association between Ki67 immunopositivity index >20% and presence of relapse ($\chi^2=18.075$, $p>0.0005$). ANOVA showed that the mean values of Ki67 index were significantly different between relapsing and non-relapsing tumours ($p=0.002$, Fig. 3b).

By univariate statistical analysis (log rank test), from all the assessed factors, tumour stage, lymph node status and Ki67 immunostaining were significant predictors of relapse ($p=0.022$, $p<0.0005$ and $p<0.0003$ respectively)

(Fig. 4). As concerns time of relapse, apart from increased Ki67 expression, lymph node positivity status and advanced stage of disease accelerated the relapse ($p=0.0005$ and $p=0.022$ respectively). Tumour transglottic location was associated with presence of recurrence but this association was of borderline statistical significance ($p=0.08$). In detail, relapse time was significantly different for the three Ki67 groups ($p<0.0003$); carcinomas overexpressing Ki67 protein (group C patients) relapsed quicker than the rest. From the Cox's proportional hazard regression analysis, the most important independent predictor of recurrence was the presence of lymph nodal metastasis ($p<0.0005$); patients with tumour-positive lymph nodes were 12.9

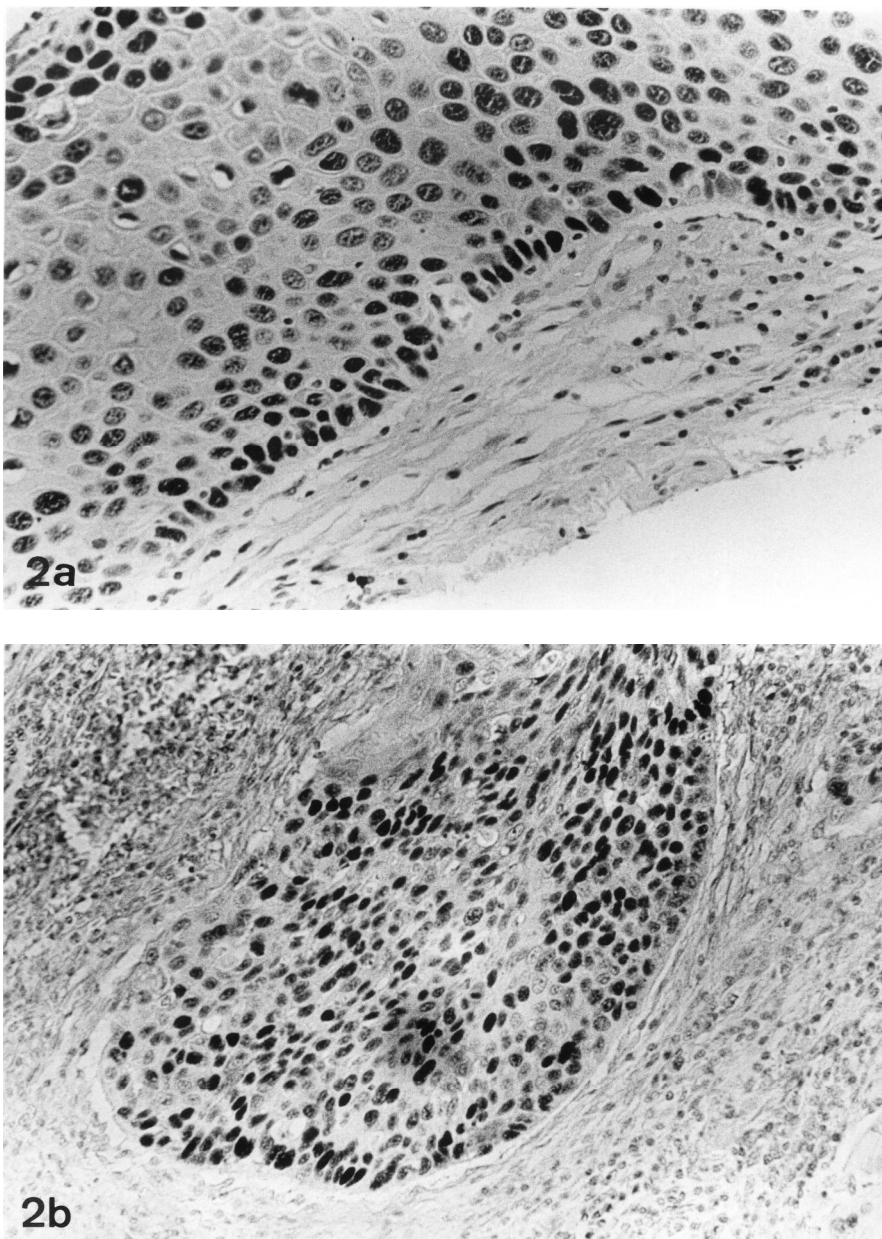


Fig. 2. a. Ki67 immunoreactivity in tumour-neighbouring dysplastic mucosa. Immunoperoxidase-ABC, x 400. **b.** Moderately differentiated squamous carcinoma. Disperse Ki67 immunopositive cancerous cells. Immunoperoxidase-ABC, x 100

times more likely to relapse than patients with negative lymph nodes. Interestingly, when lymph node status was not inserted in the model, the next most important factor influencing patient's disease-free survival was Ki67 immunopositivity group; group C was in greater risk of relapse. Actually, an increase in Ki67 index resulted in greater risk of recurrence.

TRF1 immunostaining

TRF1 was immunohistochemically detected in 53 of

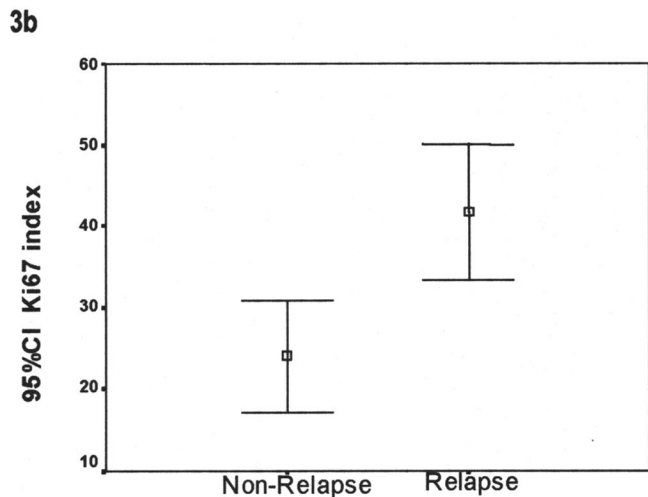
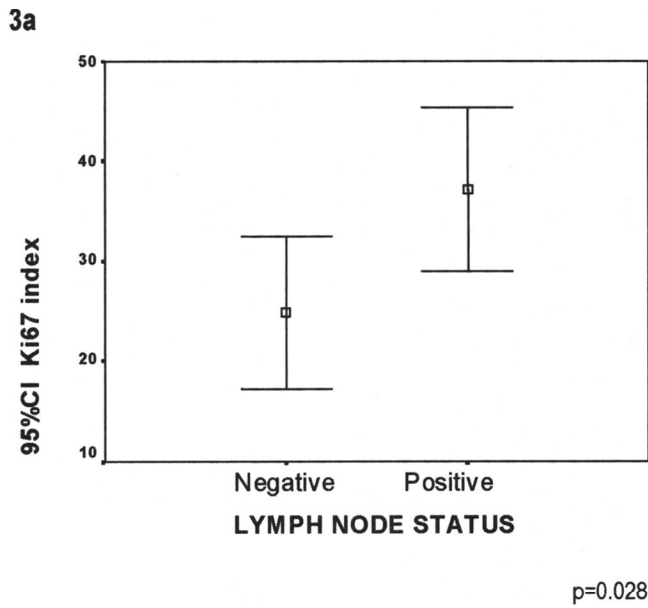


Fig. 3. a. ANOVA shows that the mean values of Ki67 index are significantly different between tumours with negative lymph nodes and tumours with metastatic lymph nodes ($p=0.028$). b. Mean values of Ki67 index differ significantly between the groups of relapsing and non-relapsing cancers (ANOVA, $p=0.002$).

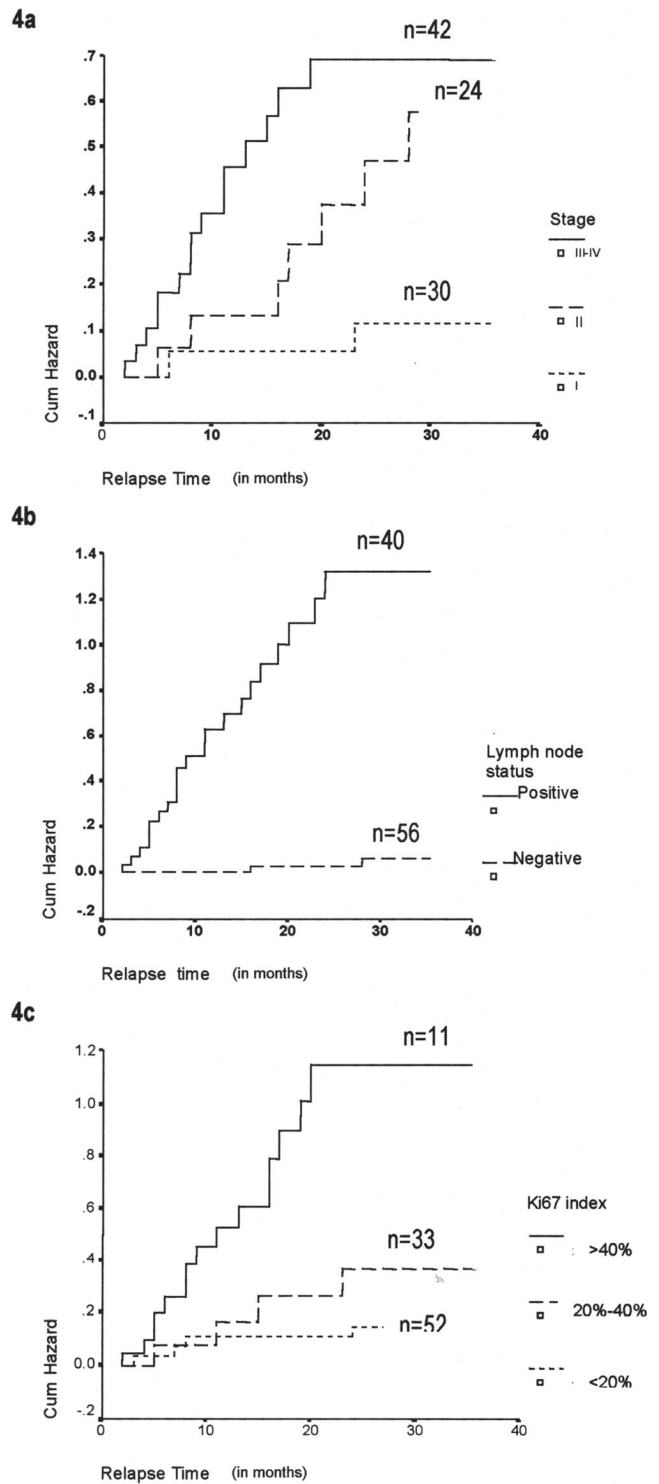


Fig. 4. Relapse time with regard to (a) tumour stage ($p=0.022$), (b) presence of lymph nodal metastases ($p<0.0005$) and (c) Ki67 immunopositivity group ($p<0.0003$) (log-rank tests).

the 96 specimens (55.2%). Tumour neighbouring dysplastic areas only rarely expressed TRF1. In the TRF1-immunopositive LSCCs, the percentages of TRF1-positive cancerous cells ranged from <1 to 48% (mean±SD: 12.048±15.522). This marker demonstrated a cytoplasmic staining pattern throughout the tumour area without any selective localization (Fig. 5). TRF1 immunopositivity status was positively linked to tumour diameter; in detail, logistic regression analysis, taking TRF1-positive expression as a dependent variable, revealed that tumour diameter was the only independent factor which contributed to TRF1-positive immunostatus and to TRF1 index ($p < 0.0005$, Table 2). Apart from the

latter statistically significant difference, neither any other variable assessed nor patients' disease-free survival or recurrence rate was in any way associated either with TRF1 immunopositivity status or with TRF1 quantitative variance in the TRF1-immunopositive samples. It is noteworthy that the two examined immunomarkers (i.e. Ki67 and TRF1) were in no way related to each other.

Discussion

The theory of carcinogenesis suggests that unlimited cell proliferation is required for development of malignant disease but cancer must attain immortality for

Table 2. The only independent factor that contributes to TRF1 quantitative expression is tumour diameter, which is in positive association with the TRF1 index (logistic regression analysis).

COEFFICIENTS ^a							
	Unstandardized Coefficients		Standardized Coefficients			95% Confidence interval for B	
	B	Std. Error	Beta	t	p value	Lower Bound	Upper Bound
Tumour Diameter	4.488	.833	.684	5.390	.000	2.794	6.182

EXCLUDED VARIABLES ^a			
Model	Beta In	t	p value
Age	-.080	-.278	.782
Location	-.075	-.330	.744
Stage	-.160	-.516	.609
Lymph node status	.090	.514	.611
Grade	-.108	-.464	.646
Nuclear big axis	-.286	-1.244	.223
Nuclear small axis	-.299	-1.310	.199
Nuclear area	-.312	-1.168	.252

^a: dependent Variable: TRF1

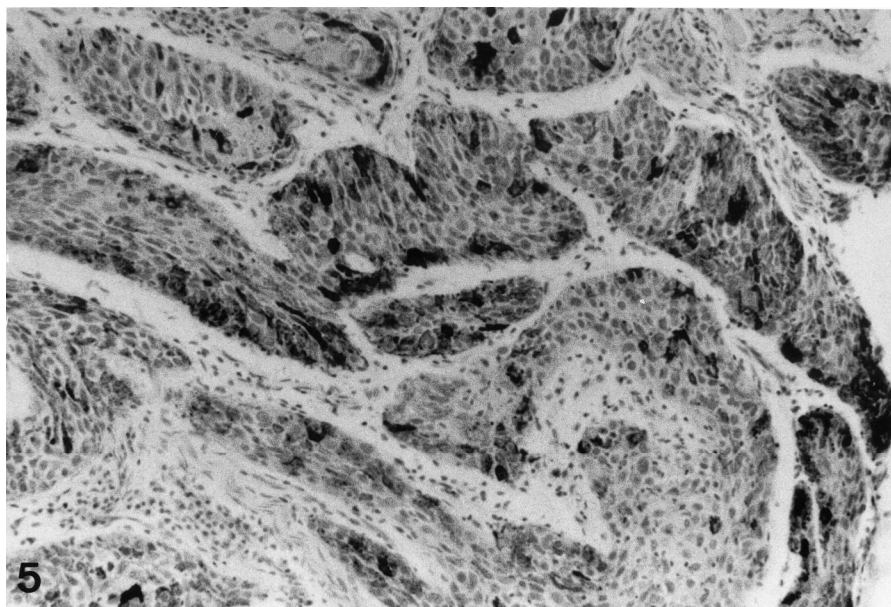


Fig. 5. TRF1 immunoreaction of strong staining intensity in malignant squamous cells. Immunoperoxidase-ABC, x 100

progression to advanced, clinically aggressive states (Koscielny et al., 2000). This concept prompted us to the comparative evaluation of a well-established immunomarker of cell proliferation (i.e. Ki67) and an indirect marker of telomerase activation (i.e. TRF1) in a series of LSCCs.

In this kind of cancer, evaluation for cervical lymph node metastasis is critical in guiding therapy and predicting prognosis (Resnick et al., 1995). In the present study, a pathological marker detected in the primary tumour site, Ki67 indice, was linked to the presence of lymph node metastasis in patients with LSCC. This significant finding reinforces the role of Ki67 as a marker of tumour aggressiveness in LSCC and is in line with Urpegui Garcia et al. (2000). However, being in discordance with previous similar articles where no such association is mentioned (Valente et al., 1999; Grzanka et al., 2000), our finding needs verification in a larger series of patients before clinicians, based on Ki67 indice, select those patients with clinically negative or equivocal necks who are most likely to benefit from cervical lymphadenectomy. Furthermore, the Ki67 indice should be re-evaluated using a monoclonal antibody which would give more reliable results and in this way any possible overestimation of Ki67 values would be avoided.

In contrast to the studies of Zidar et al. (1996), of Urpegui Garcia et al. (2000) and of Perez Garro Rio et al. (2000), in the present survey no significant association was found between tumour degree of differentiation and Ki67 expression. However, as mentioned before, Ki67 immunopositivity was localized at the basaloid neoplastic cells in keratinising LSCC. This observation indicates that the periphery of tumour islands in the latter specimens is composed of highly proliferating primitive cells which are the progenitors of the non-proliferating, more differentiated cells in the central portion. The prominence of Ki67-immunoreactive malignant cells at the tumours' invading margins, which was also frequently noticed in our samples, is probably related to the increased aggressiveness of the above cell subpopulation. Ki67 appears to be a marker of tumour cell aggressiveness not being dependent on tumour cell morphological features of differentiation.

Investigations of LSCCs agree that advanced stage and cervical node metastasis correlate with poor survival (Resnick et al., 1995); our results are in line with the above, well-established observations. Additionally, in our patients, a strongly significant relationship has been observed between the tumour's proliferative fraction, as assessed by Ki67 immunolabelling and recurrence-free survival. In agreement with a recent article (Sittel et al., 2000), Ki67 had a significant influence in recurrence-free survival, accelerating the tumour relapse. It is noteworthy that our multivariate analysis established Ki67 expression along with nodal involvement as independent prognosticators, while therapeutical treatment was being taken into account; the latter was necessary since we investigated a rather heterogenous

series encompassing cases with different pathological stages and patients who had undergone different treatment (e.g. radical or selective neck dissection). As concerns current literature, Horibe et al. (2000) found no association of Ki67 index with recurrence in early stage LSCCs while Perez Carro Rios et al. (2000), as well as Sittel et al. (2000) found statistically significant relationships between cell proliferation index and the clinical evolution of patients with LSCC.

To the best of our knowledge, this is the first immunohistochemical study of TRF1 in LSCC. This marker was expressed in a considerable proportion of our cases but it was independently-related only to increased tumour size. In contrast to telomerase activity, which is supposed to be involved early in tumourigenesis of head and neck and laryngeal squamous carcinomas (Hohaus et al., 1996; Sumida et al., 1999) and is detectable in dysplastic lesions, TRF1 immunodetection appears to be a late event in laryngeal tumourigenesis, being barely detectable in tumour-adjacent, dysplastic mucosa; this finding does raise some questions as to whether TRF1 is a marker of telomerase activity. Interestingly, tumour size was not linked with Ki67 indice in our samples. The lack of association between TRF1 expression and proliferative fraction of the examined cases implies that cell immortalization and proliferative capacity are two distinct pathways of tumour growth. It is noteworthy that no changes in the proliferation rate have been detected in TRF1-overexpressing cell lines (Shen et al., 1997), reinforcing the view that proliferation rate and TRF1 expression are not interrelated. On the other hand, not all cycling cells require telomerase activity (Sharma et al., 1996); for instance, normal differentiated cells retain the ability to proliferate and do so in the apparent absence of telomerase and, consequently, of TRF1. In our cases, increased tumour size is more likely to be due to cell immortalization, as possibly evidenced by TRF1 expression, than to an increase in the proliferative activity of the malignant cells (at least at that time of the tumour's natural history). Expression of telomerase, as indicated by TRF1 immunoreaction in almost all advanced malignancies, suggests that immortal cells are probably required to maintain tumour growth (Kim et al., 1994). Nevertheless, some neoplasms can reach a clinically significant size without immortalisation e.g. adenomata of the thyroid. Of course, definite conclusions can be made only when telomerase activity is directly detected by a sensitive molecular technique [i.e. telomeric repeat amplification protocol (TRAP) assay].

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