



MHC class I antigens and tumour-infiltrating leucocytes in laryngeal cancer: long-term follow-up

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Summary Alteration in MHC class I expression may be used by cancer cells to avoid immune destruction. Much experimental evidence supports this idea, although survival studies are very scarce. To investigate whether the presence or absence of HLA-A, -B and -C antigens in laryngeal carcinoma influences survival, a series of 60 primary laryngeal tumours treated surgically and normal tissues were evaluated in frozen sections for the expression of MHC class I antigens and tumour-infiltrating leucocytes (CD3, CD4, CD8, CD11b, CD1, CD20 and CD16), using monoclonal antibodies and the APAAP technique. Long-term follow-up from the patients is available, ranging from 6 to 10 years. Thirteen tumours presented total HLA-ABC loss, five selective losses of HLA-A antigens and one absence of HLA-B antigens. Total losses were statistically associated with several clinical and pathological parameters, but there were no differences regarding tumour-infiltrating leucocytes. After conducting a prospective study, only T and N staging and scoring according to Glanz's malignancy classification were found to be independently related to patients' outcome. From our data, we conclude that neither complete loss of HLA class I antigens nor tumour-infiltrating leucocytes appear to influence survival in squamous cell carcinoma of the larynx.

Keywords: laryngeal carcinoma; MHC antigens; tumour-infiltrating leucocytes

MHC class I antigens are membrane glycoproteins involved in effective killing by cytotoxic T cells, which require HLA class I compatibility between T cells and target cells (Thorsby, 1982; Festenstein and Garrido, 1986). Alterations in HLA-ABC expression may be one method used by cancer cells to avoid immune destruction (Garrido, 1987). Decreased HLA expression has been discovered in several human tumours, including breast, lung, skin, colorectal, gastric and laryngeal carcinomas (López-Nevot *et al.*, 1986, 1989; Redondo *et al.*, 1991; Ruiz-Cabello *et al.*, 1989). However, few studies have analysed the mechanisms responsible for such alterations. In previous papers, we investigated changes in the expression of HLA class I antigens during malignant transformation of the laryngeal epithelium and some mechanisms involved (Esteban *et al.*, 1989), and we found several clinical and pathological correlations between HLA-ABC losses and aggressiveness (Esteban *et al.*, 1990a). MHC class II antigens were only found in verrucous cell carcinomas, thus DR expression seems to be associated with tumours with excellent prognosis (Esteban *et al.*, 1990b). On the other hand, laryngeal tumours are often characterised by an inflammatory reaction that follows the growth of neoplastic elements, but its prognostic value and its functional significance remain to be determined. The aim of the present work is to present the long-term results of the series and to evaluate the prognostic significance of MHC class I antigens and tumour-infiltrating leucocytes (TILs) in patients with laryngeal carcinoma after long-term follow-up.

Materials and methods

Patients

Sixty patients with squamous cell carcinoma of the larynx were included in the study. None of them received radio-

therapy or chemotherapy before surgery. All were male. Age ranged from 44 to 75 years (average 58.68 years). Tumours were classified as originating from the supraglottic region (33.55%), glottis (18.30%), subglottis (2.33%) and pyriform sinus (3.5%). Four cases were considered transglottic (6.7%). Thus, there were 57 laryngeal and three hypopharyngeal tumours in the study. Surgical techniques consisted of cordectomy (four cases), frontolateral laryngectomy with epiglottoplasty as popularised by Harvey Tucker (two cases), supraglottic horizontal laryngectomy (12 cases) and total laryngectomy (42 cases). Neck dissection was performed at clinical stage IV disease and at stage III as indicated by the presence of or high risk of cervical metastasis and by the specific site of the primary tumour. A total of 22 patients underwent ipsilateral and 18 bilateral functional neck dissections. Radical ipsilateral neck dissection was done in three patients, and a bilateral staged procedure in four. Post-operative radiation therapy was decided on an individual basis by a multispeciality joint tumour board (otolaryngologist, oncologist and pathologist), and radiotherapy was chosen for 29 patients. Basically, radiotherapy was chosen for all T4 and/or node-positive tumours, since functional neck dissection allows pathological staging for the neck. There were four T1, four T2, 37 T3 and 15 T4 carcinomas. Forty-two patients were classified as NO, six N1, eight N2 and four N3. Follow-up ranged from 66 to 112 months, available for all patients. At present, 19 patients have died: 12 patients as a result of the disease (five neck relapses, two pharyngeal recurrences, three cases with pulmonary metastasis, one case with neck relapse and lung metastasis, one stomal recurrence) and there were seven deaths unrelated to the laryngeal disease (one myocardial infarction; 1 car crash; 1 chronic pulmonary obstructive disease; one patient committed suicide, and three died as a result of other tumours: lung, prostate and oesophagus).

Location and diameter of the primary tumour, T and N pathological staging (according to UICC, 1992 version), number of lymph nodes from the neck dissection and number of lymph node metastases, pathological global staging, blood group and Rh of the patient, age, smoking and alcohol consumption, first symptom–diagnosis intervals and follow-up (in months) were recorded for each case.

Pathological analysis

Tumours were classified into three grades by the UICC modified Broders' system (Wahi, 1972), and all were scored according to Glanz's (Glanz, 1984) and Jakobsson's (Jakobsson *et al.*, 1973) grading systems for squamous cancer. In addition, two modifications to the former were included [Crissman *et al.* (1984) and the authors' modification]. The histological grading of malignancy was based upon the tumour cell population (structure, differentiation, nuclear polymorphism and mitosis) and tumour-host relationship (mode of invasion, stage of invasion, vascular invasion and cellular response). Histological analyses were performed without any knowledge of the clinical stage, treatment or further course of the disease.

Monoclonal antibodies, alkaline immunophosphatase technique

The immunohistochemical technique and monoclonal antibodies against MHC class I antigens were described in previous publications (Esteban *et al.*, 1989, 1990). The following MAbs were included against leucocyte determinants: GRT2, which recognises the common leucocyte antigen CD45 (Huelin *et al.*, 1988); Leu 4, Leu 3a and Leu 2a, against CD3, CD4 and CD8 respectively (Becton Dickinson); Bear-1 against CD11b (Keizer *et al.*, 1985); OKT6 (Ortho) against CD1; GRM1 against CD16 (Ruiz-Cabello *et al.*, 1988); and IOM-1 against CD20 (immuno-tech).

Interpretation of immunohistological results

A tumour was classified as negative when no staining was detected in any of ten randomly chosen microscopic fields, and as positive when all tumour cells were stained in ten fields. In our series, no specimens showed a heterogeneous pattern of staining for the HLA-ABC antigens (Esteban *et al.*, 1990b). Tumour-infiltrating leucocytes were averaged after counting the number of stained cells in ten microscopic high-power fields.

Statistical analysis

We used the proportional hazards model as specified by Cox (1972). In this model, a positive regression coefficient means an increased risk of death for a given level with respect to the baseline level, whereas a negative coefficient indicates the opposite effect. Selection of the most predictive variables was based on a stepwise algorithm with a nominal significance level of 10%. ANOVA was used to compare groups of tumour-infiltrating leucocytes.

Results

Expression of HLA-A, B, C antigens

Normal laryngeal mucosa was always positive for HLA-ABC antigens. Losses of these antigens were always related to malignancy. We found 13 total losses of HLA-ABC antigens, five selective losses of HLA-A and one of HLA-B antigens.

Study of tumour-infiltrating leucocytes

Analysis of the tumour-infiltrating leucocytes was performed in most cases (50 patients). The infiltrating cells were composed of T cells and some macrophages (Table I). Langerhans cells, natural killer (NK) cells and B cells were sparse. No significant differences were found between MHC class I antigen expression and immunophenotype of TILs.

Survival analysis

The survival analysis calculated from 60 patients showed a high probability of tumour-related death for the T, N and score according to Glanz's classifications (Table II). No discriminating effect on tumour-related death, however, could be detected on the expressional status of HLA-A, B or C/ β_2m antigens. In addition, the cell composition in the infiltrates did not affect the frequency of metastasis and the 5 year survival.

Discussion

In addition to genetic alterations, some events are probably needed to acquire the malignant phenotype of a tumour. Thus, cellular malignant transformation is frequently associated with alteration in MHC antigens (Garrido *et al.*, 1993), and these alterations may profoundly affect the way in which the tumour behaves, decreasing or enhancing specific anti-tumoural immune mechanisms (Festenstein, 1987). It has been shown in several animal models that the immunogenicity of tumours depends on the expressional status of MHC class I antigens of tumour cells (Hämmerling *et al.*, 1987). In a recent study, selective growth arrest and phenotypic reversion of prostate cancer cells by phenylacetate, a non-toxic differentiation inducer, was accompanied by an increased amount of HLA transcripts (Samid *et al.*, 1993). Therefore, regulation of tumour HLA expression might be a strategy for the evasion of immune surveillance by the malignant cells.

Concerning the molecular basis of the defect in HLA class I expression in our tumours, we previously reported (Esteban *et al.*, 1989) by Southern and Northern blot analysis that transcriptional regulation of HLA expression is likely to be involved in this phenomenon. Further evidence supporting this concept includes stimulation studies with γ -interferon (IFN) in tumours with undetectable expression of HLA-A, -B or -C antigens (Ruiz-Cabello *et al.*, 1989), and modulation of MHC class I expression by oncogene activation (Versteeg *et al.*,

Table II Coefficients, standard errors and odds ratio for the Cox regression model

Variable	Coefficient	Standard error	Odds ratio
N	0.7464	0.2881	2.1094
T	1.2032	0.6551	3.3307
Glanz's score	0.7113	0.2789	2.0366

Table I Leucocytic infiltrate^a in tumours with total and selective HLA-ABC losses comparison of HLA-ABC positive tumours vs total or selective losses

CD	Tumours (number of cases studied for each subpopulation)		P-Value	HLA-ABC (-)	P-Value
	HLA-ABC (+)	Selective loss			
CD3	42.369 (35)	41.420 (5)	0.9368	34.460 (10)	0.3798
CD4	27.305 (37)	22.240 (5)	0.4599	18.336 (10)	0.0733
CD8	19.335 (37)	23.900 (5)	0.5329	21.373 (11)	0.699
CD11b	15.956 (35)	14.58 (5)	0.6908	20.54 (10)	0.0807
CD20	2.968 (31)	2.68 (5)	0.8470	2.75 (6)	0.8746
CD16	4.145 (30)	2.42 (5)	0.4116	5.21 (7)	0.5572
CD1	8.883 (36)	7.26 (5)	0.7145	4.814 (7)	0.0982

^aMeans of counting ten microscopic high-power fields.

1988). Clinical studies on the MHC expressional status of malignant tumours and survival are very scarce (Möller *et al.*, 1991; Passlick *et al.*, 1994). In our study, we showed that total loss of HLA-A, -B and -C antigens in laryngeal carcinomas is not a prognostic factor. However, series including a greater number of patients may improve the level of statistical significance, as univariate analysis has shown a strong relationship between HLA class I total loss and tumour aggressiveness (Esteban *et al.*, 1990b).

On the other hand, we have not found any significant difference between class I expression and the immunophenotype of infiltrating cells, and also between these TILs and survival. The exact role that lymphocytes may play against laryngeal cancer is unknown. There are few papers investigating the role of TILs in head and neck cancer, and results are inconclusive (Hiratsuka *et al.*, 1984; Rabin *et al.*, 1984; Harabuchi *et al.*, 1985; Wolf *et al.*, 1986; Böheim *et al.*, 1987; Zeromski *et al.*, 1986; Guo *et al.*, 1987). In breast and colon tumours, two reports performed multivariate analysis and they found that the amount of lymphocytic infiltrate was independently predictive of the risk of death (Marques *et al.*, 1990; Kubota *et al.*, 1992), although they did not measure the immunophenotype of this infiltrate. Others have shown T cells to be a favourable prognostic sign, whereas B cells are generally not predominant in various human cancers (Shimokawara *et al.*, 1982). In contrast, some reports show evidence that tumours are stimulated into growth by host cells in the tumour stroma (Stewart and Tsai, 1993). In this context, *in vitro* studies demonstrated that tumour-infiltrating leucocytes display an immune inhibitory effect (Fulton, 1987; Vose and Bonnard, 1982). Other authors report important functional deficiencies in T cells (Gorski *et al.*, 1994; Fu *et al.*, 1989).

Concerning Langerhans cells, a high density of these cells has been considered of favourable prognosis for patients with squamous cell carcinoma of the larynx in the univariate analysis (Gallo *et al.*, 1991). However, we found a very small proportion of Langerhans cells in our cases with no influence on prognosis.

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