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Prognostic significance and clinical relevance of the expression of the HER family of type I receptor tyrosine kinases in human laryngeal squamous cell carcinoma

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ABSTRACT

Introduction: The full clinical relevance of the expression pattern of HER family of type I receptor tyrosine kinases in laryngeal squamous cell carcinoma remains to be elucidated. We evaluated the clinical relevance of such parameter in our population.

Patients and methods: This study examined the expression pattern of HER family receptor members by quantitative immunohistochemistry and the amount of the EGF binding sites by a radioligand binding assay, in the same group of 67 LSCC patients, analysing the correlation between the expression of the four HER receptors and the clinical and prognostic parameters.

Results: HER1 levels inversely correlated with that of HER2–4, while HER2–4 directly correlated among them. Cox univariate analysis using HER1–4 values as continuous covariates indicated that HER1 expression was directly associated with the risk of death and relapse while that of HER2–4 was inversely associated with the risk of death. Among the patients with high HER1 expressing tumours, those with tumours co-expressing HER2–4 showed a lower risk of death and relapse (in particular regional relapse) than those with tumours displaying a negative HER2–4 status.

Conclusions: The evaluation of HER2–4 status adds more power to the prognostic role of HER1 detection. In the era of molecularly targeted therapy, the expression of HER family of receptor tyrosine kinases in LSCC may hold relevant clinical significance and turn out to be a key factor in prognostic assessment and in treatment planning.

1. Introduction

The HER family of type I receptor tyrosine kinases is comprised of four members: HER1 (ErbB1), HER2 (ErbB2/Neu), HER3 (ErbB3), and HER4 (ErbB4).[1] Their activation relies on the binding of agonists, mainly growth factors as EGF and TGF-α, which induces receptor homo- and heterodimerisation, intrinsic tyrosine kinase activation, receptor auto-phos-

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phorylation as well as phosphorylation of several downstream targets.[1–3] HER receptors activate signal transduction pathways including Ras-Raf-Erk, STAT3 and STAT5 and phosphoinositide 3-kinase/Akt-mTOR cascades, which regulate cellular responses, such as proliferation, differentiation and survival.[1–3] The different affinity of the various agonists to the various HER dimers produces a great combinatorial complexity, diversifying biological responses to different ligands (as EGF and neuregulin families).[1,2,4,5]

The clinical relevance of the HER receptors in human cancers is unquestionable.[2] Overexpression of HER1 occurs in more than 90% of head and neck squamous cell carcinoma (HNSCC) and correlates with more advanced tumours, poor survival, and higher risk of lymph node metastasis.[6–12] Nevertheless, the role of the other HER proteins in head and neck carcinogenesis and/or as prognostic markers has been evaluated with contradictory results and still need to be better elucidated.[13–20]

As the HER receptors act as homo- and heterodimers with different affinities for the various ligand, their functional state is the result of a complex biochemical balance among the concentration of the proteins in the various compartments of the cells and the concentrations of their ligands. Therefore, to better clarify the carcinogenic as well as the prognostic role of the single HER receptor, we evaluated the pattern of expression of HER family as a whole.[21,22] We perform our study in a series composed exclusively by laryngeal squamous cell carcinomas (LSCC), to reduce the heterogeneity deriving from the different sites of origin. We assessed, in the same group of 67 LSCC patients, the expression of HER receptors and the amount of the EGF-binding sites and we correlated their expression to clinical parameters and patient relapse-free, metastasis-free and overall survival.

2. Materials and methods

2.1. Patients

This study was a retrospective analysis of archival biopsies from 67 untreated consecutive primary LSCC patients admitted to the Department of Otolaryngology of the Catholic University, Rome, between 1999 and 2003. Histological grading and TNM classification were performed on conventional paraffin sections according to the recommendations of the International Union Against Cancer. Accordingly, tumours were graded as well- (G1), moderately- (G2) or poorly- (G3–G4) differentiated. All patients in this study received standard therapeutic management including, as for the primary, radical surgical resection of the primary tumour by a total or partial laryngectomy according to the primary lesion; as for the neck, we performed therapeutic comprehensive neck dissection when there was lymph node involvement at clinical presentation and observation, according to the "wait and see" policy, under strict follow-up conditions in cN0 necks. Adjuvant radiotherapy (60–70 Gy, 180 cGy per fraction) + concomitant chemotherapy (q21 cisplatin) on primary and nodal echelons was administered in case of locally advanced tumours (pT4), positive or close resection margins, extra-nodal spread, pN2–N3 disease. All the patients in our study have been treated according to this standard protocol. The majority of patients (57/67, 85.1%) were in cN0 stage (clinically negative neck node tumours). None of the patients received preoperative chemotherapy or radiotherapy. The median follow-up period was 47 months (range: 10–85 months).

2.2. Immunohistochemistry

Tumour tissues obtained at surgery, were fixed in formalin and paraffin-embedded according to standard procedures. Consecutive tissue sections, 4 μm thick, of representative blocks from each case, were deparaffinised in xylene, rehydrated, treated with 0.3% H_2O_2 in methanol for 10 min to block endogenous peroxidase activity, and subjected to heat-induced epitope retrieval in microwave oven using the Dako ChemMate detection kit (Dako, Milano, Italy) according to the manufacturer's instructions. Slides, from all cases studied, were then simultaneously processed for immunohistochemistry on the DAKO autostainer, universal staining system (Dako) using the Vectastain ABC peroxidase kit (Vector Laboratories, Burlingame,CA). Endogenous biotin was saturated by a biotin-blocking kit (VectorLaboratories). Sections were incubated with antibodies against HER1 (clone H11, dilution 1:150, Dako), HER2 (Dilution 1:150, Dako), HER3 (RTJ.2 Dilution 1:200, Santa Cruz Biotechnology, Inc., Heidelberg, Germany), HER4 (C-18, dilution 1:200, Santa Cruz Biotechnology, Inc.), cytokeratins (CK) 14 and 17 (clone LL002 and E3, dilution 1:100, Novocastra, Newcastle-upon-Tyne, UK), proliferating cell nuclear antigen (PCNA, clone PC-10, dilution 1:200, DAKO) for 1 h. Negative controls were performed using non-immunised rabbit and mouse serum, omitting the primary antibodies. We evaluated qualitative features of the staining for different HER proteins as the prevalent site of localisation (membrane, cytoplasm, nucleus).

2.3. Quantitative immunohistochemistry

The intensity of immunohistochemical staining was evaluated using image analysis based on Photoshop (version 6.0; AdobeSystems, San Jose, CA) together with 'The image-processing toolkit' (version 3.0, 1998, CRC Press, Boca Raton, FL) according to the method previously reported[23] with some modifications. The technical setup included a Zeiss Axioskop microscope (Zeiss; Jena, Germany) equipped with a Nikon Coolpix 950 digital camera (Nikon Corporation; Tokyo, Japan). Three ×20 superimposable fields were chosen from consecutive sections so as to best reflect the overall immunostaining of the tumour sections. Tumoural tissue was manually selected in digitalised images and the integrated density (ID) of the immunostaining was calculated as the product of the mean density value of the immunoreactive regions by the percentage of the immunostained tumour tissue. The computerised image analysis of all tissue sections were done by three pathologists without prior knowledge of the clinical and pathological parameters.

CK14, CK17 and PCNA immunostainings, performed on 55 cases, were independently evaluated by two pathologists, without prior knowledge of the clinical and pathological parameters, by counting positive cells × 100 number of negative cells in three microscopic fields at 400× magnification.

The data were recorded as negative (<25% of positive cells in the tumour) or positive (⩾25% of positive cells in the tumour).

2.4. Epidermal growth factor receptor radioligand-binding assay

RBA of EGF was performed on 67 laryngeal SCC as previously reported.[12] Briefly, tissue specimens were frozen on dry ice shortly after surgical removal and stored at –80 °C until processed. A representative section of each specimen was retained for histological examination to ascertain that most of the cells were neoplastic.

The membrane pellet was resuspended in 25 mM Tris, 1.5 mM EDTA, 5 mM NaN3, 20% glycerol, and 10 mM MgCl2. Aliquots of the suspension (100 μl containing 300–500 μg of protein) were incubated with ^{125}I-EGF (2.6 nM; 800,000 Ci/mmol; NEN, DuPont, Wilmington, DE) in the presence or absence of unlabelled EGF (1 μM) for 12–16 h at room temperature in a final volume of 400 μl. Binding was blocked by the addition of 3 ml of ice-cold 25 mM Tris, 20% glycerol, 5 mM NaN_3, and 0.1% BSA. After centrifugation at 2000*g* for 20 min at 0 °C, the supernatant was carefully aspirated, and pellets were counted in a gamma counter. Results were expressed as fmoles/mg of protein.

2.5. Statistical analysis

For some statistical evaluation, different cut-off values of HER1–4 IDs (mean or median value) were tested in the survival analyses. In the case of HER4, two different immunostaining patterns ('nuclear' or 'cytoplasmic') were observed, anyway the ID was calculated as the overall (nuclear + cytoplasmic) immunostaining of the cells. Although all cut-off values tested behaved as significant discriminators, the best prognostic cut-off point value was the mean value of HER1–4 IDs. Nonparametric Spearman's rho was used to analyse the correlations between HER expression levels. Mann–Whitney or Kruskall-Wallis tests were used to analyse the distribution of HER family member receptors status according to various clinico-pathological parameters. Survival data were available for all the 67 patients. The Cox-Mantel method was used to evaluate the prognostic role of HER family receptor IDs and EGF-binding sites as continous variables. All medians and life tables were computed using the product-limit estimate by Kaplan and Meier, and the curves were examined by means of the log-rank test. Univariate and multivariate analysis was performed by Cox's proportional hazards model. Relapse-free survival was calculated from the date of first surgery to that of clinical loco-regional recurrence. Overall survival was calculated from the date of first surgery to that of death. All *p*-values were two-sided. All statistical analyses were done by JMP 7 statistical software (SAS Institute Inc., Cary, NC).

3. Results

3.1. HER1–4-immunostaining patterns

In LSCC neoplastic cells HER1 immunoreactivity was mainly localised at the periphery of the cytoplasms, in the more external cells of the neoplastic nest; HER2 immunostaining was mainly present in the cytoplasm of the centrally located tumour cells; HER3 was mainly observed in the nuclear compartment of the centrally located neoplastic cells. Two different expression patterns of HER4 were observed: one was characterised by a prominent nuclear immunoreactivity in the majority of the neoplastic cells and by a less intense cytoplasmic staining (here defined as 'nuclear pattern'); the other, was characterised by a weak nuclear staining in less than 25% of the cells and by a faint cytoplasmic immunostaining (here defined as 'cytoplasmic pattern'). In LSCC, nuclear and cytoplasmic patterns were present in different areas of the same tumours although in different proportions. For a practical purpose, we defined tumours as having 'nuclear pattern' or 'cytoplasmic pattern' on the grounds of the pattern type present in more than 50% of the tumoural area examined.

3.2. Correlation of HER1–4 expression to EGF binding

EGF binding, as detected by RBA, directly correlated with the amount of HER1 ($p = 0.0001$) and inversely with that of HER2 ($p = 0.009$), HER3 ($p = 0.0006$) and HER4 ($p = 0.02$), as detected by IHC. Moreover, the amount of HER1 was also inversely correlated with that of HER2 ($p = 0.0001$) HER3 ($p = 0.0001$) and HER4 ($p = 0.003$), while HER2–4 directly correlated among them.

Tumours expressing low levels of HER2 and/or HER3 and/or HER4 contained significantly higher amounts of EGFR than those expressing high levels of HER2 and/or HER3 and/or HER4 (fmoles/mg of membrane protein: 33.1 + 5.6 S.E., $n = 20$ vs. 9.9 + 3.9 S.E., $n = 47$; $p = 0.02$).

3.3. Clinico-pathological correlations and survival analysis of HER1–4 expression in LSCC

Based on the cut-off points the HER1, HER2, HER3 and HER4 positive cases were 23/67 (34.3%), 29/67 (43.3%), 34/67 (50.8%) and 29/67 (43.3%), respectively.

The distribution of HER1–4 ID levels according to clinico-pathological parameters was reported in Table 1. Only HER3 ID levels inversely correlated with T-classification and lymph node involvement, while the other receptor IDs did not correlate with any clinical parameter at diagnosis.

With a median follow-up of 47 months, cervical lymph-node metastases occurred in 18 of 67 patients (26.9%). At the end of the study, 21 of 67 patients (31.3%) had died of cancer.

Cox proportional hazards model indicated that the risk ratios of death, related to unit increase in the HER ID levels, were: 1.035 (95% CI: 1.01–1.05; $p = 0.0025$), 0.95 (95% CI: 0.90–0.99; $p = 0.021$), 0.83 (95% CI: 0.69–0.95; $p = 0.0041$) and 0.96 (95% CI: 0.93–0.99; $p = 0.0059$) for HER1–4, respectively. Furthermore, the risk ratio of metastases were 1.04 (95% CI: 1.0–1.1; $p = 0.0002$), 0.93 (95% CI: 0.87–0.98; $p = 0.0053$), 0.79 (95% CI: 0.61–0.93; $p = 0.0018$) and 0.96 (95% CI: 0.93–0.99; $p = 0.0087$), for HER1–4, respectively.

The survival curves according to the HER1–4 status showed that a shorter overall and regional metastasis-free survival was associate to high HER1 (>14.1 ID) or, on the con-

Table 1 – Distribution of HER1, HER2, HER3 and HER4 levels according to clinico-pathological parameters.

	# Cases	HER1	HER2	HER3	HER4
Total	67	6.5 (0.2–78.0)[a]	12.9 (0.1–48.5)	2.8 (0–40.4)	36.9 (12.7–73.6)
Age (years)					
<60	26	5.2 (0.2–40.0)	13.0 (1.6–45.7)	3.7 (0.2–21.1)	37.7 (13.6–71.0)
>60	41	8.0 (0.5–78.0)	12.9 (0.1–48.5)	2.3 (0–40.4)	34.6 (12.7–73.6)
Tumour site					
Glottic	6	7.6 (0.2–30.1)	9.5 (1.8–24.2)	3.7 (0.2–5.7)	35.8 (15.5–71.0)
Supraglottic	23	8.5 (0.2–78.0)	10.5 (0.2–48.5)	2.9 (0–31.5)	38.4 (12.7–69.8)
Transglottic	38	6.0 (0.3–72.0)	13.6 (0.1–41.9)	2.5 (0–40.4)	36.9 (13.6–73.6)
T-classification					
1	11	5.0 (0.2–47.7)	12.5 (1.9–36.6)	4.6 (1.3–31.5)	36.9 (16.7–71.0)
2	20	7.1 (0.2–78.0)	10.2 (0.1–39.7)	2.7 (0–21.1)	37.1 (12.7–67.1
3	21	6.0 (0.5–52.4)	14.3 (1.6–48.5)	3.1 (0.4–40.4)	39.1 (13.6–69.2)
4	15	4.4 (3.0–72.3)	10.0 (2.0–32.3)	1.6 (0–9.0)[b]	34.6 (16.1–73.6)
Nodal status					
Negative	57	6.4 (0.2–78.0)	13.5 (0.1–48.5)	3.1 (0–40.4)	36.9 (12.7–73.6)
Positive	10	11.4 (3.9–72.3)	7.0 (1.8–32.3)	1.2 (0–8.1)[c]	36.0 (15.5–67.1)
Tumour grading					
1	10	6.7 (0.3–32.3)	30.5 (5.4–39.7)	2.4 (0.5–31.5)	44.2 (19.0–71.0)
2	17	5.5 (0.2–72.3)	13.7 (2.0–41.9)	4.0 (0–40.4)	33.0 (15.6–68.7)
3	40	8.0 (0.2–78.0)	10.2 (0.1–48.5)	2.3 (0–21.1)	38.8 (12.7–73.6)
Clinical staging					
I	10	8.5 (0.2–47.7)	9.5 (1.9–29.5)	4.1 (1.3–10.7)	38.0 (16.7–71.0)
II	13	8.5 (2.6–78.0)	9.6 (0.2–39.7)	2.1 (0–21.1)	33.0 (12.7–53.9)
III	26	6.0 (0.2–52.4)	15.5 (0.1–48.5)	3.1 (0–40.0)	38.7 (13.6–69.8)
IV	18	11.4 (3.0–72.3)	8.8 (1.8–32.3)	1.6 (0–9.0)	36.0 (15.5–73.6)

[a] The results are expressed as integrated density values (I.D.); Median (range).
[b] Kruskal–Wallis test: $p = 0.03$.
[c] Mann–Whitney test: $p = 0.02$.

trary, to low HER2 (<15), HER3 (<5.2) and HER4 (<39.2) IDs (Figs. 1 and 2).

The correlation of HER2–4 status with patient survival may be biased by the inverse correlation between the expression of HER1 and that of the other HER receptors coupled with the strong association of HER1 levels with the overall and metastases-free survival rates. Then, we evaluated whether the adjustment for HER2–4 status of the tumours added information to the relationship between HER1 levels and the overall and metastases-free survival rates. The plots of the Cox proportional hazards model estimates of the overall and regional metastasis-free survival as a function of HER1 ID level, for patients with HER2–4 positive or negative tumours, were shown in Figs. 1 and 2, respectively. At median follow-up, the estimated proportions of surviving patients, at the cut-off value of HER1 (14 ID), were 63.8% and 29.2% for those with HER2–4 positive and negative tumours, respectively. Relative to the metastasis-free survival, the estimated proportions of metastasis-free patients were 85.3% and 35.7% for those with HER2–4 positive and negative tumours, respectively.

As revealed by the univariate analysis, cases with high T classification, lymph node involvement, high tumour grade, HER1 or EGFR positive tumours, HER2–4 negative tumours, were associated with an increased risk of death (Table 2). The risk of metastasis was associated with lymph node involvement, HER1 or EGFR positive tumours and HER2–4 negative tumours. In the multivariate analysis, lymph node involvement and HER1 positive tumours retained an independent negative prognostic significance, relative to the overall survival. HER2–4 negative tumour status and, in a lesser degree, HER1 positive status, retained an independent negative prognostic significance relative to the metastasis-free survival (Table 3).

As revealed by the Kaplan–Meier analyses, patients with tumours expressing the HER4 nuclear pattern had a significantly longer metastase-free (Mantel-Cox test, $p = 0.0006$) and overall survival (Mantel-Cox test, $p = 0.0007$) than those with tumours expressing the HER4 cytoplasmic pattern.

The expression of HER1–4 was associated with that of CK-14 basal-layer cytokeratin and PCNA. In particular, CK-14 status of the tumours was directly associated with the expression of HER1 ($p = 0.0007$) and inversely related with that of HER2 ($p = 0.009$), HER3 ($p = 0.019$) and HER4 ($p = 0.018$). PCNA status of the tumours was directly associated with the expression of HER1 ($p = 0.0043$) and inversely related with that of HER2 ($p = 0.006$), HER3 ($p = 0.007$) but not of HER4 ($p = 0.76$).

4. Discussion

HNSCC are a heterogeneous group of tumours arising at different anatomical regions with largely variable clinical behaviours.[24] In the present study we decided to study exclusively squamous cell carcinomas of the larynx (LSCC), which the American Cancer Society classifies as a part of the respiratory system, separately from oral cavity and pharynx.[25] Several peculiarities can be highlighted, that suggest the need to study laryngeal cancer as a separate entity, both from a clinical and a molecular[26] point of view. Under a clinical point of

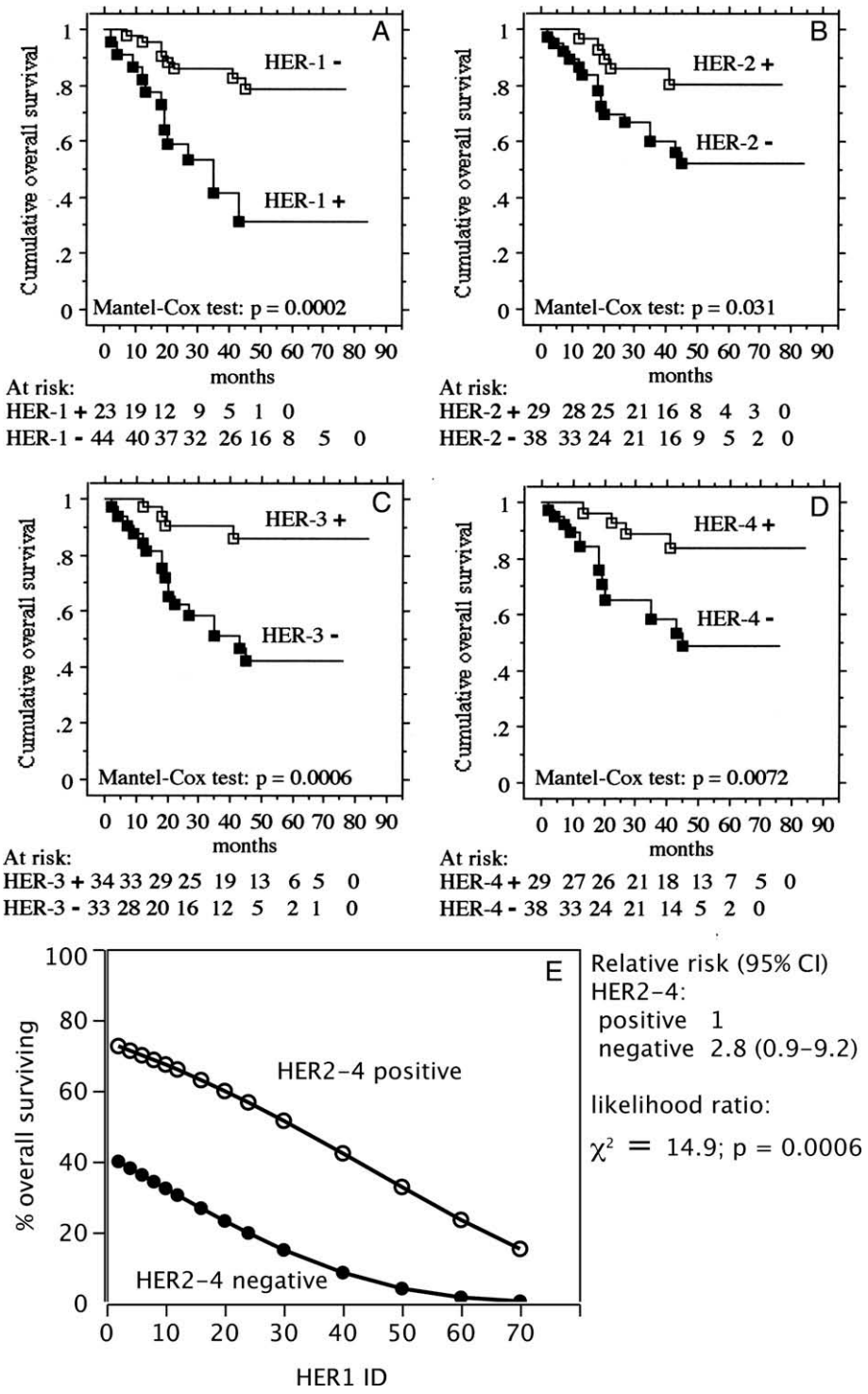

Fig. 1 – Overall survival according to the HER 1–4 status in LSCC patients (A–D). Plots of the estimates of overall survival as a function of the levels of HER1 ID for patients with HER2–4 positive or negative tumours (E).

view, differently from the other head and neck subsites, the male/female ratio is markedly higher and LSCC did not have a significant improvement in the 5-year survival rates during the last 30 years.[25] In fact, among the most frequent malignancies, cancers of the larynx and of the uterine corpus are the only ones without an increase in the 5-year survival rates during the last 30 years.[25] As for LSCC, we can identify several potential reasons for this failure.[27] One of these is that the TNM, which remains so far the only consistent clinical predictors for disease control and disease-specific survival in LSCC,[28,29] leads to an inadequate prognostic stratification. In fact similar patients, affected by tumours with similar clinico-pathological parameters and undergoing the same treatment, may differ widely in prognosis. This is probably due

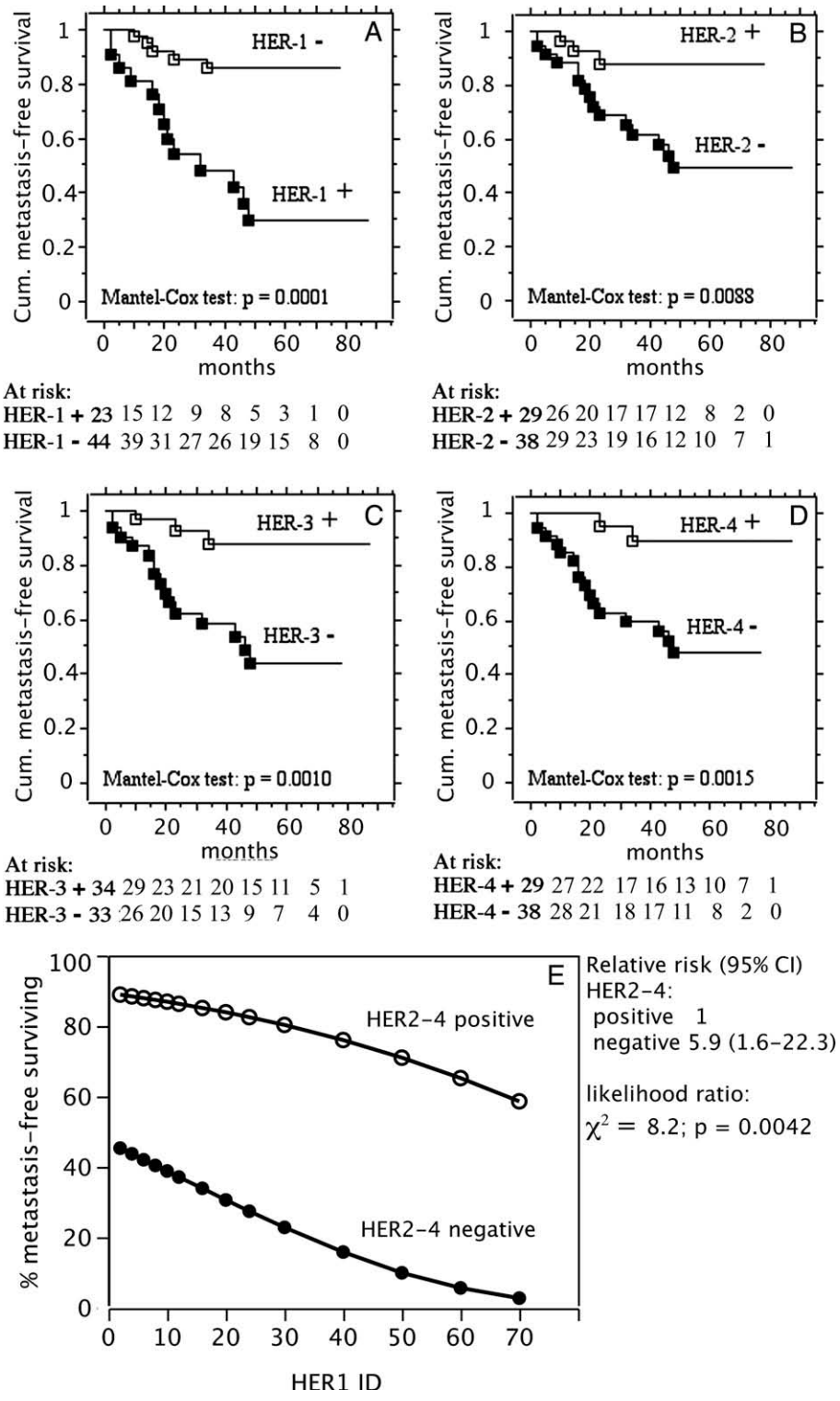

Fig. 2 – Regional metastasis-free survival according to the HER1–4 status in LSCC patients (A–D). Plots of the estimates of metastasis-free survival as a function of the levels of HER1 ID for patients with HER2–4 positive or negative tumours (E).

to the extreme biological heterogeneity of LSCCs and contributes to a lack of consistency in treatment planning.

The molecular characterisation by the study of molecular predictive factors is aimed to overcome such lack of consistency, defining homogeneous groups of patients for prognostic stratification and treatment selection. Even if a plethora of reports have tried to evaluate their potential clinical role, no molecular marker contributes at present to the clinical decision-making process. Nevertheless there is a provocative evidence supporting a strong role for EGFR expression and for its ligand, TGFa, in predicting prognosis, because it adversely influences overall, relapse-free and metastasis-free survival

Table 2 – Univariate analysis of prognostic covariates in laryngeal squamous cancer patients.

	Overall survival			Nodal metastasis-free survival		
Covariates	RR[a]	CI (95%)[b]	P	RR	CI (95%)[b]	P
Age						
<60	1			1		
>60	0.9	0.4–1.7	0.9	0.9	0.4–2.1	0.8
Tumour site						
Supraglottic	1			1		
Transglottic	0.9	0.4–1.8	0.8	1.2	0.5–2.9	0.7
T-classification						
1–2	1			1		
3–4	3.6	1,7–7.5	0.0008	1.6	0.7–3.6	0.3
Nodal status						
Negative	1			1		
Positive	3.5	1.6–7.7	0.002	3.3	1.3–8.6	0.013
Tumour grading						
G1	1			1		
G2–G3	2.3	1.1–4.6	0.02	1.5	0.6–3.5	0.3
HER1 (I.D.)						
<14.1	1			1		
>14.1	4.6	1.9–11.2	0.0008	6.7	2.4–18.9	0.0003
HER2 (I.D.)						
>15.1	1			1		
<15.1	2.8	1.0–7.8	0.04	4.5	1.3–15.6	0.02
HER3 (I.D.)						
>2.8	1			1		
<2.8	5.4	1.8–16.1	0.002	6.2	1.8–21.5	0.004
HER4 (I.D.)						
>39.2	1			1		
<39.2	4.0	1.3–11.8	0.01	7.5	1.7–32.6	0.007
EGFR (fmoles/mg of protein)						
<8.39	1			1		
>8.39	1.8	1.2–2.6	0.0015	7.4	0.9–59.4	0.012
HER2-4 status						
Positive	1			1		
Negative	4.6	1.9–12.2	0.0006	10.4	3.4–31.9	0.0001

[a] Unadjusted relative risk.
[b] 95% confidence intervals.

as well, in LSCC, independently of treatment (surgery, chemotherapy and/or radiation)[8,11,12,30–32] and makes in our opinion EGFR the most reliable prognostic molecular marker at present for laryngeal SCC.[27] The ability of predicting regional recurrences, in particular, is extremely interesting under a clinical perspective, as nodal relapse is an ominous event and is the most frequent cause of failure and disease related death in head and neck oncology in general and in laryngeal oncology in particular.

The present study confirms the prognostic significance of HER1 (EGFR) expression, which is negatively correlated with overall and relapse-free (in particular metastasis free) survival (Figs. 1 and 2). On the other side our data show that the expression of the other HER proteins inversely correlates with HER1 expression and has a protective role, as a high expression is associated with a longer overall and relapse-free survival. As for HER3 and HER4 receptor our results confirm some clinical studies conducted so far, that suggested that their expression is associated with a favourable prognosis in several malignancies[18–20] as squamous cell carcinomas of the oral cavity,[33] breast ductal carcinomas,[34] meningiomas[35] and bladder cancer.[36] Furthermore, the presence of HER4 reduced the recurrence in breast HER2 expressing cancers[37] and a significantly longer survival was observed in patients with bladder cancers expressing high levels of HER2, when co-expressed with high levels of HER3 and HER4.[38]

In LSCC, similarl to breast cancer,[39] both 'nuclear' and 'cytoplasmic' patterns of HER4 immunostaining can be evidenced. We observed that patients with LSCC showing the HER4 'nuclear' pattern, which in breast cancer is associated with lower grading,[39] had, on the contrary, a significantly longer relapse-free and overall survival.

It has been observed that the proliferation markers, PCNA and Ki67, are inversely associated with disease-free and overall survival in LSCC patients.[40,41] Our findings that, in LSCC, PCNA levels were directly associated with HER1 ID and inversely related to HER2 and HER3 IDs, further support the protective role of HER2–4 in LSCC.

These data confirm distinct roles for HER receptor family members and may reveal useful, as the assessment of HER2–3-4 levels in the group of high risk patients with tumours expressing high HER1levels, integrates the predictive power of EGFR expression itself, both for overall and for metastasis-free survival, as shown in Figs. 1 and 2. Another

Table 3 – Multivariate analysis of prognostic covariates in laryngeal squamous cancer patients.

	Overall survival			Nodal metastasis-free survival		
Covariates	RR[a]	CI (95%)[b]	P	RR	CI (95%)	P
Age						
<60	1			1		
>60	0.8	0.4–2.3	0.7	0.8	0.3–2.5	0.8
Tumour site						
Supraglottic	1			1		
Transglottic	0.9	0.3–2.7	0.9	0.6	0.2–2.0	0.4
T-classification						
1–2	1			1		
3–4	1.3	0.4–4.3	0.7	1.3	0.4–4.2	0.6
Nodal status						
Negative	1			1		
Positive	3.1	1.0–9.2	0.042	2.5	0.7–9.3	0.1
Tumour grading						
G1	1			1		
G2–G3	1.9	0.5–6.4	0.3	1.0	0.3–2.9	0.3
HER1 (I.D.)						
<14.1	1			1		
>14.1	7.7	2.1–28.9	0.002	3.4	0.9–12.7	0.05
HER2–4 status						
Positive	1			1		
Negative	1.2	0.4–4.1	0.7	6.7	1.6–28.0	0.009

[a] Unadjusted relative risk.
[b] 95%confidence intervals.

way to introduce the assessment of expression profile of HER proteins in the clinical practice may be to frame the tumours in one of the two gross HER protein expression clusters with significantly different survival rates.

Finally also the localisation of HER4 gives further prognostic information, so that the evaluation of the global expression pattern of HER proteins may be a more useful tool than the EGFR expression alone, in the clinical perspective of molecular characterisation.[27] The immediate consequence would be a better treatment selection avoiding, for example 'under-treatment', such as a 'wait and see' policy on cN0 necks, in case of LSCCs with an unfavourable HER expression profile.

On the other hand, our results may help to explain the variable responses shown to drugs acting on the EGFR pathway, which has been demonstrated to be a good molecular target for the treatment of LSCC.[42,43] In fact, based upon the promising results of the use of C225 monoclonal anti-EGFR antibody concomitantly with radiotherapy,[44] and on its relatively favourable toxicity profile, we are now experimenting it in a clinical trial on patients with supraglottic cancer, for an organ preservation intent. Such protocol seems effective but some laryngeal SCCs have a lower response to C225 even if they overexpress HER1. Since HER proteins as HER2 and HER3 have already been demonstrated to be involved in the sensitivity to drugs targeting the HER1 pathway,[45] the different patterns of expression of HER proteins may be evaluated in relation to C225 with clinical response.

Conflict of interest statement

None declared.

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REFERENCES

1. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* 2001;**2**:127–37.
2. Olayioye MA, Neve RM, Lane HA, Hynes NE. The ErbB signaling network: receptor heterodimerization in development and cancer. *EMBO J* 2000;**19**:3159–67.
3. Schlessinger J. Common and distinct elements in cellular signaling via EGF and FGF receptors. *Science* 2004;**306**:1506–7.
4. Burden S, Yarden Y. Neuregulins and their receptors: a versatile signaling module in organogenesis and oncogenesis. *Neuron* 1997;**18**:847–55.
5. Tzahar E, Pinkas-Kramarski R, Moyer JD, et al. Bivalence of EGF-like ligands drives the ErbB signaling network. *EMBO J* 1997;**16**:4938–50.
6. Riesterer O, Milas L, Ang KK. Use of molecular biomarkers for predicting the response to radiotherapy with or without chemotherapy. *J Clin Oncol* 2007;**25**:4075–83.
7. Rafferty MA, Fenton JE, Jones AS. An overview of the role and inter-relationship of epidermal growth factor receptor, cyclin D and retinoblastoma protein on the carcinogenesis of squamous cell carcinoma of the larynx. *Clin Otolaryngol Allied Sci* 2001;**26**:317–20.
8. Rubin GJ, Melhem MF, Gooding WE, et al. Levels of TGF-alpha and EGFR protein in head and neck squamous cell carcinoma and patient survival. *J Natl Cancer Inst* 1998;**90**:824–32.

9. Gale N, Kambic V, Poljak M, et al. Chromosomes 7,17 polysomies and overexpression of epidermal growth factor receptor and p53 protein in epithelial hyperplastic laryngeal lesions. *Oncology* 2000;**58**:117–25.
10. Watanabe H, Suzuki T, Ito Y, et al. Prognostic significance of EGFR and p53 expression, and angiogenesis in laryngeal squamous cell carcinoma. *Nippon Jibiinkoka Gakkai Kaiho* 1999;**102**:243–53.
11. Almadori G, Cadoni G, Galli J, et al. Epidermal growth factor receptor expression in primary laryngeal cancer: an independent prognostic factor of neck node relapse. *Int J Cancer* 1999;**84**:188–91.
12. Maurizi M, Almadori G, Ferrandina G, et al. Prognostic significance of epidermal growth factor receptor in laryngeal squamous cell carcinoma. *Br J Cancer* 1996;**74**:1253–7.
13. Weinstein GS, Nuamah IF, Tucker J, Montone K. Evaluation of HER-2/neu (c-erbB-2) oncogene expression in whole organ sections of supraglottic squamous cell carcinoma. *Ann Otol Rhinol Laryngol* 1996;**105**:275–9.
14. Tantawy A, Youins L, Hamza M. Expression of c-erb B-2 oncoprotein in cancer of the larynx in relation to invasion of the cartilagenous framework and prognosis. *Eur Arch Otorhinolaryngol* 1999;**256**:72–7.
15. Cavalot A, Martone T, Roggero N, et al. Prognostic impact of HER-2/neu expression on squamous head and neck carcinomas. *Head Neck* 2007;**29**:655–64.
16. Krecicki T, Jelen M, Zalesska-Krecicka M. C-erbB-2 immunostaining in laryngeal cancer. *Acta Otolaryngol* 1999;**119**:392–5.
17. Kazkayasi M, Hucumenoglu S, Siriner GI, Hucumenoglu M. Over-expression of p53 and c-erbB-2 oncoproteins in laryngeal carcinoma. *Eur Arch Otorhinolaryngol* 2001;**258**:329–35.
18. Thybusch-Bernhardt A, Beckmann S, Juhl H. Comparative analysis of the EGF-receptor family in pancreatic cancer: expression of HER-4 correlates with a favourable tumor stage. *Int J Surg Investig* 2001;**2**:393–400.
19. Suo Z, Risberg B, Kalsson MG. Et al. EGFR family expression in breast carcinomas. c-erbB-2 and c-erbB-4 receptors have different effects on survival. *J Pathol* 2002;**196**:17–25.
20. Rotterud R, Nesland JM, Berner A, Fossa SD. Expression of the epidermal growth factor receptor family in normal and malignant urothelium. *BJU Int* 2005;**95**:1344–50.
21. Xia W, Lau YK, Zhang HZ, et al. Combination of EGFR, HER-2/neu, and HER-3 is a stronger predictor for the outcome of oral squamous cell carcinoma than any individual family members. *Clin Cancer Res* 1999;**5**:4164–74.
22. Bei R, Budillon A, Masuelli L, et al. Frequent overexpression of multiple ErbB receptors by head and neck squamous cell carcinoma contrasts with rare antibody immunity in patients. *J Pathol* 2004;**204**:317–25.
23. Ranelletti FO, Almadori G, Rocca B, et al. Prognostic significance of cyclooxygenase-2 in laryngeal squamous cell carcinoma. *Int J Cancer* 2001;**95**:343–9.
24. Shah JP, Patel KJ. *Head and neck surgery and oncology*. 3rd ed. Mosby Ltd.; 2003.
25. Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2009. *CA Cancer J Clin* 2009;**59**:225–49.
26. Bosch FX, Ritter D, Enders C, et al. Head and neck tumor sites differ in prevalence and spectrum of p53 alterations but these have limited prognostic value. *Int J Cancer* 2004;**111**:530–8.
27. Almadori G, Bussu F, Cadoni G, et al. Molecular markers in laryngeal squamous cell carcinoma: Towards an integrated clinicobiological approach. *Eur J Cancer* 2005;**41**:683–93.
28. Shah JP, Karnell LH, Hoffman HT, et al. Patterns of care for cancer of the larynx in the United States. *Arch Otolaryngol Head Neck Surg* 1997;**123**:475–83.
29. Licitra L, Bernier J, Grandi C, et al. Cancer of the larynx. *Crit Rev Oncol Hematol* 2003;**47**:65–80.
30. Maurizi M, Scambia G, Benedetti PP, et al. EGF receptor expression in primary laryngeal cancer: correlation with clinico-pathological features and prognostic significance. *Int J Cancer* 1992;**52**:862–6.
31. Wen QH, Miwa T, Yoshizaki T, et al. Prognostic value of EGFR and TGF-alpha in early laryngeal cancer treated with radiotherapy. *Laryngoscope* 1996;**106**:884–8.
32. Dassonville O, Formento JL, Francoual M, et al. Expression of epidermal growth factor receptor and survival in upper aerodigestive tract cancer. *J Clin Oncol* 1993;**11**:1873–8.
33. Ekberg T, Nestor M, Engstrom M, et al. Expression of EGFR, HER2, HER3, and HER4 in metastatic squamous cell carcinomas of the oral cavity and base of tongue. *Int J Oncol* 2005;**26**:1177–85.
34. Lee Y, Cho S, Seo JH, et al. Correlated expression of erbB-3 with hormone receptor expression and favorable clinical outcome in invasive ductal carcinomas of the breast. *Am J Clin Pathol* 2007;**128**:1041–9.
35. Andersson U, Guo D, Malmer B, et al. Epidermal growth factor receptor family (EGFR, ErbB2–4) in gliomas and meningiomas. *Acta Neuropathol* 2004;**108**:135–42.
36. Memon AA, Sorensen BS, Melgard P, et al. Expression of HER3, HER4 and their ligand heregulin-4 is associated with better survival in bladder cancer patients. *Br J Cancer* 2004;**91**:2034–41.
37. Barnes NL, Khavari S, Boland GP, et al. Absence of HER4 expression predicts recurrence of ductal carcinoma in situ of the breast. *Clin Cancer Res* 2005;**11**:2163–8.
38. Memon AA, Sorensen BS, Meldgaard P, et al. The relation between survival and expression of HER1 and HER2 depends on the expression of HER3 and HER4: a study in bladder cancer patients. *Br J Cancer* 2006;**94**:1703–9.
39. Srinivasan R, Gillett CE, Barnes DM, Gullick WJ. Nuclear expression of the c-erbB-4/HER-4 growth factor receptor in invasive breast cancers. *Cancer Res* 2000;**60**:1483–7.
40. Liu M, Lawson G, Delos M, et al. Prognostic value of cell proliferation markers, tumour suppressor proteins and cell adhesion molecules in primary squamous cell carcinoma of the larynx and hypopharynx. *Eur Arch Otorhinolaryngol* 2003;**260**:28–34.

[41]. Bradford CR, Wolf GT, Carey TE, et al. Predictive markers for response to chemotherapy, organ preservation, and survival in patients with advanced laryngeal carcinoma. *Otolaryngol.Head Neck Surg*. 1999;**121**:534–8.

42. Mendelsohn J, Baselga J. The EGF receptor family as targets for cancer therapy. *Oncogene* 2000;**19**:6550–65.
43. Lango MN, Shin DM, Grandis JR. Targeting growth factor receptors: integration of novel therapeutics in the management of head and neck cancer. *Curr Opin Oncol* 2001;**13**:168–75.
44. Bonner JA, Harari PM, Giralt J, et al. Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. *New Engl J Med* 2006;**354**:567–78.
45. Erjala K, Sundvall M, Junttila TT, et al. Signaling via ErbB2 and ErbB3 associates with resistance and epidermal growth factor receptor (EGFR) amplification with sensitivity to EGFR inhibitor gefitinib in head and neck squamous cell carcinoma cells. *Clin Cancer Res* 2006;**12**:4103–11.