Prognostic significance of epidermal growth factor receptor in laryngeal squamous cell carcinoma

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Summary Epidermal growth factor receptor (EGFR) content was determined by a radioligand receptor assay in 140 primary laryngeal squamous cell carcinomas (median value of 8.4 fmol mg⁻¹ protein, range 0–169.9 fmol mg⁻¹ protein). Cox univariate regression analysis using EGFR as a continuous variable showed that EGFR levels are directly associated with the risk of death (χ² = 14.56, P-value = 0.0001) and relapse (χ² = 7.77, P-value = 0.0053). A significant relationship between EGFR status and survival was observed at the different arbitrary cut-off values chosen (8, 16 and 20 fmol mg⁻¹ protein). The cut-off value of 20 fmol mg⁻¹ protein was the best prognostic discriminator. In fact, the 5 year survival was 81% for patients with EGFR⁺ tumours compared with 25% for patients with EGFR⁻ tumours (P < 0.0001). The 5 year relapse-free survival was 77% for patients with EGFR⁺ tumours compared with 24% for patients with EGFR⁻ tumours (P < 0.010). When clinicopathological parameters and EGFR status were examined in the multivariate analysis, T classification and EGFR status retained an independent prognostic value. In this study we demonstrated that high EGFR levels single out patients with poor prognosis in laryngeal cancer.

Keywords: epidermal growth factor receptor; squamous cell carcinoma; larynx; prognosis

Laryngeal cancer accounts for 1.2% of all new cases of malignancy in the United States (Silverberg et al., 1990) with an incidence of 12 300 cases per year, and accounts for 0.7% of all cancer deaths.

At present, therapy is determined by age, performance status, stage of disease and tumour location. However, these clinical parameters are still inadequate for the prognostic characterisation of laryngeal cancer, since patients with identical clinicopathological features may differ widely in the development of the disease and in response to therapy (Snow, 1989). Thus, the identification of factors more strictly related to tumour cell biology may be useful in characterising patients with a different prognosis. Previous studies have identified biological factors, such as DNA index and/or ploidy (Riu et al., 1991; Kearsley et al., 1991), or cell proliferation markers (Coltrera, 1993), which may predict the clinical outcome of laryngeal cancer. Moreover, much attention has been focused on the role of oncogenes, i.e. p53, c-ras, c-myc (Brennan et al., 1995; Anderson et al., 1992; Scambia et al., 1994a; Irish and Bernstein, 1993) and EGFR expression and/or amplification (Irish and Bernstein, 1993; Miyaguchi et al., 1990; Scambia et al., 1991; Santini et al., 1991).

The epidermal growth factor/epidermal growth factor receptor (EGF/EGFR) system may be involved in cell transformation through different mechanisms, the most frequent of which are autocrine overproduction of epidermal growth factor/transforming growth factor alpha (EGF/ TGFα) and overexpression of normal EGFR (by gene amplification or altered transcriptional mechanisms) (Velu, 1990). Tumours containing high EGFR levels appear to have a worse prognosis in breast (Sainsbury et al., 1987), ovarian (Scambia et al., 1992), endometrial (Scambia et al., 1994b), bladder (Neal et al., 1991) and oesophageal (Ozawa et al., 1989) cancer.

Although the role of EGFR in the development of the malignant laryngeal squamous cell phenotype has been described (Granda and Teweard, 1993; Stanton et al., 1994), the prognostic role of EGFR in laryngeal tumours is not fully established. A previous study (Dassonneville et al., 1993) reported an independent prognostic role of high EGFR levels in a series of head and neck cancers. In our preliminary study, despite the relatively small number of laryngeal tumours examined and the short follow-up time, EGFR levels are associated with a more aggressive laryngeal cancer behaviour (Maurizi et al., 1992). In this study the clinical significance of EGFR in laryngeal squamous cell carcinoma was investigated for the first time on a large single institution patient population with a long follow-up.

Materials and methods

Our study included 140 primary laryngeal cancer patients admitted to the Department of Otolaryngology of the Catholic University of Rome. All patients were staged according to TNM classification (Hermanek and Sobin, 1992) and tumours graded as well (G1), moderately (G2) and poorly (G3) differentiated. The clinicopathological features of the patients are listed in Table I.

Tumours were classified as supraglottic, glottic or subglottic when the extent of disease did not permit identification of the original site.

Seventy-three patients underwent radical laryngectomy and sixty-seven had conservative surgery (i.e. cordectomy, horizontal supraglottic laryngectomy and hemilaryngectomy). None of the patients received preoperative chemotherapy or radiotherapy. All the patients with relapse or regional neck metastasis underwent salvage surgery or irradiation.

EGFR assay

Tissue specimens were frozen on dry ice shortly after surgical removal and stored at –80°C until processed. A representative section of specimens was retained for histological examination, which revealed that most of the cells were cancer cells.

The membrane fraction and cytosol were prepared as described elsewhere (Iacobelli et al., 1987; Scambia et al., 1991). The membrane pellet was resuspended in TENG plus 10 mM magnesium chloride. Aliquots of the suspension
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(100 µl containing 300 to 500 µg protein) were incubated with 

\[^{125}\text{I}]\text{EGF (NEN Dupont DE Nemours) (3.2 nM) in the presence or absence of unlabelled EGF (1 µM) for 16 h at room temperature in a final volume of 400 µl. Binding was stopped by the addition of 3 ml of TENG plus 0.1% bovine serum albumin (BSA). Pellets were obtained by centrifugation at 2000 \times g for 20 min at 0°C and counted in a gamma counter for 1 min. Protein concentration was measured by the Bradford method (Bradford, 1976). Results were expressed as fmol per mg of membrane protein (fmol mg\(^{-1}\) protein). Receptor characterisation has been reported elsewhere (Scambia et al., 1991).

Table 1 Distribution of EGFR levels according to clinicopathological parameters in 140 primary laryngeal cancer patients

| Parameter | No. | Median (fmol mg\(^{-1}\) protein) | Range |
|-----------|-----|---------------------------------|-------|
| Total     | 140 | 8.4                             | 0–169.9 |
| Sex       |     |                                 |       |
| Males     | 130 | 8.3                             | 0–169.9 |
| Females   | 10  | 10.6                            | 0–149.9 |
| Age       |     |                                 |       |
| <60 years | 46  | 7.2                             | 0–86.9 |
| >60 years | 94  | 9.1                             | 0–169.9 |
| Tumour site |   |                                 |       |
| Glottic   | 13  | 6.1                             | 0–64.8 |
| Supraglottic | 47 | 8.5                             | 0–169.9 |
| Transglottic | 80 | 8.7                             | 0.5–164.8 |
| T Classification | | | |
| 1         | 22  | 8.8                             | 0–64.8 |
| 2         | 57  | 7.2                             | 0.3–169.9 |
| 3         | 41  | 8.3                             | 0–86.9 |
| 4         | 20  | 14.3                            | 1.1–164.8 |
| Lymph-node involvement | | | |
| No        | 103 | 7.6                             | 0–169.9 |
| Yes       | 37  | 10.5                            | 2.37–164.8 |
| Histopathological grading | | | |
| G1        | 28  | 7.5                             | 0–64.8 |
| G2        | 71  | 9.1                             | 0–164.8 |
| G3        | 41  | 8.4                             | 0–169.9 |

Statistical analysis

The Wilcoxon rank sum non-parametric test was used to analyse the distribution of EGFR levels according to clinicopathological characteristics. The Cox–Mantel method was used to evaluate the prognostic role of logarithmically transformed EGFR values as a continuous variable (Cox, 1972).

Different cut-off values for EGFR were tested in the survival analysis and arbitrary values of 8, 16 and 20 fmol mg\(^{-1}\) protein were chosen. All medians and life tables were computed using the product-limit estimate by Kaplan and Meier (1958) and the curves were examined by means of the log-rank test (Mantel, 1966). Multivariate analysis was performed by the Cox proportional hazards model (Cox, 1972). Relapse-free survival (RFS) was calculated from the date of first surgery to the date of clinical or pathological local recurrence. Overall survival (OS) was calculated from the date of first surgery to the date of death (median follow-up was 49 months, range 2–84 months).

Results

The distribution of EGFR levels in 140 primary laryngeal cancer patients is shown in Table 1. EGFR levels ranged from 0 to 169.9 fmol mg\(^{-1}\) protein, with a median value of 8.4 fmol mg\(^{-1}\) protein. Using arbitrary cut-off values of 8, 16 and 20 fmol mg\(^{-1}\) protein, 53%, 26% and 20% of tumours, respectively, were considered EGFR\(^+\). No difference in EGFR distribution in relation to sex, age, tumour site, T classification, lymph node involvement or histopathological grading was observed (Table 1). During the follow-up period local recurrence was observed in 50 cases. At the end of the study 37 patients had died of cancer. Cox univariate regression analysis using EGFR as a continuous variable showed that EGFR levels are directly associated with the risk of death (\(\chi^2=14.56, P\text{-value}=0.0001\)) and relapse (\(\chi^2=7.77, P\text{-value}=0.0053\)).

Figure 1 shows the survival curves according to EGFR status. A significant relationship was found between EGFR positivity and a shorter survival at the different cut-off values chosen. The cut-off value of 20 fmol mg\(^{-1}\) protein was the

![Figure 1](image-url) Survival rate according to EGFR status in 140 primary laryngeal cancer patients: overall survival (37 patients had died), relapse-free survival (50 patients had local recurrence).
best prognostic discriminator. In fact, the 5 year survival was 81% (95% confidence intervals, CI 74–88%) for patients with EGFR+ tumours compared with 25% (95% CI 5–45%) for patients with EGFR− tumours (P<0.0001). Similarly, the relapse-free survival curves shown in Figure 1 demonstrated that EGFR+ patients have a shorter RFS than EGFR− patients at different cut-off values tested. At the cut-off value of 20 fmol mg−1 protein the 5 year relapse-free survival was 77% (95% CI 68–86%) for patients with EGFR+ tumours compared with 24% (95% CI 2–46%) for patients with EGFR− tumours (P<0.010). T classification was also significantly correlated with survival in the univariate analysis (Table II).

Table III shows the multivariate analysis of prognostic variables for survival in laryngeal cancer patients. T classification and EGFR status are the most important independent prognostic factors in overall and relapse-free survival. Moreover, in overall survival, histopathological grading seems to have an additional role as a prognostic factor.

**Table II** Univariate analysis of prognostic variables for survival in 140 primary laryngeal cancer patients

| Prognostic variable | No. of patients | Five year survival % | 95% CI | P-value | Five year survival % | 95% CI | P-value |
|---------------------|-----------------|----------------------|--------|---------|----------------------|--------|---------|
| Age                 |                 |                      |        |         |                      |        |         |
| <60 years           | 46              | 77                   | 64–90  | 0.059;  | 55                   | 38–72  | NS      |
| ≥60 years           | 94              | 66                   | 55–77  | NS      | 61                   | 49–73  | NS      |
| Tumour site         |                 |                      |        |         |                      |        |         |
| Glottic             | 13              | .a                   | .a     | .a      | 85                   | 65–105 | NS      |
| Supraglottic        | 47              | 67                   | 53–81  | NS      | 55                   | 38–72  | NS      |
| Transglottic        | 80              | 66                   | 53–79  | NS      | 55                   | 42–68  | NS      |
| T classification    |                 |                      |        |         |                      |        |         |
| 1–2                 | 78              | 84                   | 75–93  | <0.0546 | 71                   | 59–83  | 0.0047  |
| 3–4                 | 62              | 52                   | 37–67  | <0.0001 | 40                   | 24–56  | <0.010  |
| Lymph node involvement |             |                      |        |         |                      |        |         |
| No                  | 103             | 73                   | 63–83  | NS      | 57                   | 46–68  | NS      |
| Yes                 | 37              | 62                   | 46–78  | NS      | 64                   | 48–80  | NS      |
| Histopathological grading |       |                      |        |         |                      |        |         |
| G1–G2               | 99              | 73                   | 63–83  | NS      | 56                   | 44–68  | NS      |
| G3                  | 41              | 62                   | 47–77  | NS      | 64                   | 49–79  | NS      |
| EGFR status         |                 |                      |        |         |                      |        |         |
| <8 fmol mg−1 protein | 66           | 85                   | 76–94  | 0.0029  | 68                   | 55–80  | 0.04    |
| ≥8 fmol mg−1 protein | 74           | 54                   | 40–68  |         | 52                   | 38–66  |         |
| EGFR status         |                 |                      |        |         |                      |        |         |
| <16 fmol mg−1 protein | 103        | 87                   | 80–94  | <0.0001 | 66                   | 56–76  | <0.049  |
| ≥16 fmol mg−1 protein | 37          | 30                   | 10–50  | <0.0001 | 31                   | 9–53   | <0.049  |
| EGFR status         |                 |                      |        |         |                      |        |         |
| <20 fmol mg−1 protein | 112        | 81                   | 74–88  | <0.0001 | 77                   | 68–86  | <0.010  |
| ≥20 fmol mg−1 protein | 28          | 25                   | 5–45   | <0.0001 | 24                   | 2–46   |         |

*For overall survival analysis of the glottic tumour site was not included because very few events occurred in this subgroup. aGlottic vs supraglottic P=0.059; glottic vs transglottic P=0.07; supraglottic vs transglottic P=NS.

**Table III** Multivariate analysis of prognostic variables for survival in primary laryngeal cancer patients

| Prognostic variable | RR   | Overall survival | P-value | RR | Relapse-free survival | P-value |
|---------------------|------|------------------|---------|----|------------------------|---------|
| Age                 |      |                  |         |    |                        |         |
| <60 years           | 2.30 | 1.02–5.16        | 4.11    | 0.0425 | 1.14                    | 0.61–2.14 | 1.19 | 0.27 |
| ≥60 years           |      |                  |         |    |                        |         |
| Tumour site         |      |                  |         |    |                        |         |
| Supraglottic        | 0.44 | 0.20–0.94        | 4.40    | 0.0357 | 0.58                    | 0.29–1.16 | 2.31 | 0.12 |
| Transglottic        | 4.75 | 2.06–10.93       | 13.44   | 0.0002 | 2.73                    | 1.36–5.47 | 8.06 | 0.0045 |
| T classification    |      |                  |         |    |                        |         |
| 1–2                 | 1.85 | 0.88–3.87        | 2.69    | 0.10 | 1.00                    | 0.50–1.98 | 0.010 | 0.90 |
| 3–4                 |      |                  |         |    |                        |         |
| Lymph node involvement |    |                  |         |    |                        |         |
| No                  | 2.93 | 1.38–6.21        | 7.84    | 0.0051 | 1.47                    | 0.73–2.95 | 1.19 | 0.27 |
| Yes                 |      |                  |         |    |                        |         |
| Histopathological grading |    |                  |         |    |                        |         |
| G1–G2               | 4.00 | 2.00–8.00        | 15.49   | 0.0001 | 2.17                    | 1.17–4.02 | 6.11 | 0.0134 |
| G3                  |      |                  |         |    |                        |         |
| EGFR status         |      |                  |         |    |                        |         |
| <20 fmol mg−1 protein | 4.00 | 2.00–8.00        | 15.49   | 0.0001 | 2.17                    | 1.17–4.02 | 6.11 | 0.0134 |
Discussion

Experimental evidence showed a role of EGFR in the development of laryngeal cancer. Our previous study reported higher EGFR levels in laryngeal tumours than in normal mucosa (Scambia et al., 1991) in accordance with other authors (Santini et al., 1991). A significant correlation between EGFR levels and stage was found in head and neck cancer (Santini et al., 1991; Dassonville et al., 1993), whereas we reported higher EGFR expression in poorly differentiated (G3) than in well/moderately differentiated (G1–G2) laryngeal tumours (Scambia et al., 1991).

To our knowledge, this is the first study analysing the prognostic significance of EGFR in a large prospective series of laryngeal squamous cell carcinomas with a long follow-up period. In our series the presence of high EGFR levels is significantly correlated with a poor overall and relapse-free survival. Moreover, analysis of logarithmically transformed EGFR values shows that the risk of death and relapse increases with increasing EGFR values in a significant way.

In the multivariate analysis, EGFR status retained an independent prognostic role, suggesting that EGFR assessment, together with clinical parameters, such as stage of disease, may improve the prognostic characterization of laryngeal cancer patients. Our data are in accordance with those of Dassonville et al. (1993) who reported a prognostic role for EGFR in head and neck tumours, and with our preliminary results on a smaller series of laryngeal cancer (Maurizi et al., 1992).

This study suggests that the assessment of EGFR status at the time of initial surgery may identify a subset of patients with a particularly poor prognosis and permit therapy to be modified accordingly. What is more, the prognostic significance of EGFR levels might imply that laryngeal cancer is a candidate for a novel anti-cancer therapy based on drugs targeted directly against EGFR activity, such as anti-EGFR monoclonal antibodies and the specific inhibitor of the EGFR tyrosine kinase, which inhibit the growth of cancer cells in vitro and in vivo (Moreshige et al., 1993; Kurachi et al., 1991; Schnurch et al., 1994; Fry et al., 1994).

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