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Prognostic value of the epidermal growth factor receptor (EGRF) and p53 in advanced head and neck squamous cell carcinoma patients treated with induction chemotherapy

Ricardo Hitt [a,*], Eva Ciruelos [a], María L. Amador [a], Amparo Benito [b], José J. Sanchez [a], Claudio Ballestin [b], Hernán Cortes-Funes [a]

[a] *Department of Medical Oncology, Hospital Universitario Doce de Octubre, Cordoba Km 5.4, Madrid 28041, Spain*
[b] *Department of Pathology, Hospital Universitario Doce de Octubre, Madrid 28041, Spain*

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Abstract

We measured the expression of the p53 nuclear protein and epidermal growth factor receptor (EGFR) in 46 biopsy samples from patients with advanced head and neck cancer treated with induction combination chemotherapy of 5-fluorouracil, cisplatin, and paclitaxel. Tumour expression of p53 protein was analysed with the monoclonal D07 antibody and EGFR with monoclonal H11 antibody. The overall response, defined as complete (CR) and partial response (PR) rates to treatment, was 88%. p53 positive staining was significantly more frequent in patients who did not respond to the induction treatment. EGFR expression failed to show any correlation with the response rate. Multivariate analysis indicated that a tumour location in the oral cavity together with p53 expression combined with moderate-to-high EGFR staining were independent prognostic factors of a shorter disease-free survival (DFS). Location of the tumour in the oral cavity and EGFR expression had independent prognostic value for overall survival (OS). We conclude that the EGFR status and an oral cavity location of the tumour have independent prognostic value in patients with advanced head and neck carcinoma treated with induction chemotherapy. The p53 status appears to be a determinant of the tumour chemo-sensitivity in advanced head and neck squamous cell carcinoma (HNSCC). The presence in the tumour of a p53-positive stain and moderate-to-high staining of EGFR is associated with a shorter DFS and time to treatment failure (TTF) probably reflecting a more aggressive tumour phenotype.

Keywords: Epidermal growth factor receptor; p53; Head and neck cancer; Prognosis

1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide [1]. More than 60% of patients present with locally advanced disease [2]. The use of induction chemotherapy in patients with advanced HNSCC is being intensively evaluated [3]. Although randomised trials have failed to show a survival advantage with the use of induction chemotherapy, patients who achieve complete response (CR) have a more favourable prognosis [4,5], suggesting that the use of induction chemotherapy may benefit only a subgroup of patients. The identification of factors that predict response to induction chemotherapy would allow the selection of patients with advanced HNSCC who would benefit from this treatment. Although

* Corresponding author. Tel.: +34 91 390 8349; fax: +34 91 460 3310.
E-mail address: rhitt@h12o.es (R. Hitt).

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several prognostic factors have been proposed, tumour node metastasis (TNM) stage at diagnosis remains the most important factor identified to date in the prognostic evaluation of HNSCC patients [6]. However, it is of note, that there is significant prognostic variation within patients with the same TNM stage [7] which reflects the heterogeneity of the tumour as well as the limitations of the currently available prognostic markers.

With our evolving understanding of the mechanisms underlying the pathology of HNSCC, a number of molecular markers have been proposed as determinants of prognosis and the response to treatment [7,8]. One of these markers is the nuclear phosphoprotein, p53, that restricts cell proliferation by inducing growth arrest and/or apoptosis [9]. Mutations in the p53 suppresser gene are among the most common genetic abnormalities in HNSCC [10]. The protein product is normally present at very low levels, almost undetectable by immunohistochemistry, but mutation of the p53 gene leads to an accumulation of mutant (non-functional) protein that is not degraded normally [11]. The high frequency of p53 mutations in HNSCC has prompted a number of studies to determine the prognostic significance of p53 in this disease. However, the results to date are contradictory and no clear correlation between p53 status and HNSCC prognosis has been found in patients treated with induction therapy [12,13]. Another potential marker is the epidermal growth factor receptor (EGFR), a 170–180 kD *trans*-membrane glycoprotein that represents one of the most important growth-regulatory signal-transduction molecules [14]. Of note, it is overexpressed in 80% to 90% of HNSCC tumours [15]. This overexpression has been shown to be an independent prognostic indicator in HNSCC patients [16–20]. Several studies have shown a correlation between EGFR status and response to different chemotherapy regimens, and to radiation therapy [15–17,19,21]. However, the role of EGFR status as a factor predictive of response to induction chemotherapy in regimens using paclitaxel has not been evaluated.

One of the principal problems in interpreting the prognostic significance of these molecular markers is the heterogeneity observed with regard to patients' characteristics, as well as to the treatment administered. In this study, our aim was to determine whether the expression of p53 protein and EGFR has any impact on response-to-treatment and clinical outcome. The study sample was a group of patients receiving homogenous treatment in a phase II trial of paclitaxel, cisplatin, and 5-fluorouracil (PPF regimen) and administered in combination as induction therapy. The results of this trial in terms of efficacy and toxicity have been reported elsewhere in Ref. [22].

2. Materials and methods

2.1. Patient selection and study design

A phase II clinical trial using induction chemotherapy was designed for patients with locally advanced HNSCC [22]. To be eligible for the study, patients had to have histologically proven squamous cell carcinoma of the larynx, hypopharynx, oropharynx, and/or oral cavity; to have a Karnofsky performance status (PS) of 70% or more; to have measurable disease; and to have adequate major organ function. All patients gave their written informed consent prior to enrollment into the study. The scientific review board and the ethics committee of our institution granted protocol approval, which included issues related to the management of biological samples. All patients had a complete medical history and physical examination and these details have been described previously in Ref. [22].

2.2. Treatment

The PPF regimen consisted of a 6-day course of paclitaxel (175 mg/m^2 as a 3 h infusion on day 1), cisplatin (100 mg/m^2 as a 1 h infusion on day 2) and 5-fluorouracil (500–750 mg/m^2/day as a 120 h continuous infusion on days 2–6). The cycle was repeated every 3 weeks for a maximum of three cycles and the treatment was administered on an outpatient basis.

2.3. Evaluation of tumour response

Patients were evaluated with respect to eligibility, staging, and treatment planning by a multidisciplinary team that included clinical oncologists, head and neck surgeons, and radiologists. The primary endpoints of the study were tumour response and toxicity. Tumour response was defined for each patient according to World Health Organization (WHO) criteria and based on the combined findings of computerised tomographic (CT) scans and physical examinations. A consensus post-induction treatment schedule for each patient was developed by the multidisciplinary team based on clinical and radiographic data related to primary site of the disease, as well as to neck node metastases.

2.4. Assessment of treatment outcome

The primary endpoints of the study were tumour response to the induction therapy, as well as the toxicity of the regimen. The criteria used to evaluate CR, PR, no change (NC), and progressive disease (PD) were based on the standard definitions established by the WHO [23]. Toxicity was graded according to the National Cancer Institute-Common Toxicity Criteria (NCI-CTC). Immunohistochemical analyses were performed

independently of the clinical data. The clinical and the pathology investigators were blinded with respect to each other's findings until the conclusion of the study.

2.5. Immunohistochemical methods

Before commencement of the therapy, fresh tumour specimens were obtained by core tissue biopsy (0.6 mm × 3–4 mm), performed by a cytopathologist at the Department of Pathology of the *Hospital Doce de Octubre*. The samples were then formalin-fixed and paraffin-embedded. All retrieved blocks were freshly cut to provide representative tumour sections which were subsequently used in the staining experiments. The following, well-characterised, mouse monoclonal antibodies were used for the present study: anti-p53 monoclonal antibody Dako (clone DO7, Dako Corp, Glostrup, Denmark); anti-EGFR monoclonal antibody Dako (clone H11, Dako Corp, Glostrup, Denmark). The antibodies were used at a final dilution of 1:100 according to the manufacturer's instructions. A mouse monoclonal antibody of the same subclass as the primary antibody was used as a negative control at a similar working dilution. Sections were immersed in boiling citric acid (0.01%; 15 min) to enhance antigen retrieval. On cooling, they were incubated with primary antibody overnight at 4 °C. Biotin-labelled horse anti-mouse immunoglobulin G antibodies were applied for 1 h, followed by avidin-biotin peroxidase complexes for 30 min. Diaminobenzidine was used as the final chromogen, and haematoxylin was used as the nuclear counter-stain.

2.6. Statistical methods

Nuclear immunoreactivity was classified on a continuous scale with values that ranged from undetectable levels (0%) to homogenous staining (100%). The two markers were measured as a percentage of positively stained tumour cells and dichotomised as discrete variables; the cut-off point for p53 being set at 50%. This was based on our previous analysis in head and neck cancer that had revealed a strong association between p53 point mutations and p53 nuclear accumulation in 50% of the tumour cells [24]. The degree of EGFR staining was assessed quantitatively in each sample by image analysis using the following score: (0) undetectable levels; (+) 10–30% membrane immunoreactivity; (++) 30–60% membrane immunoreactivity; (+++) >60% membrane immunoreactivity [25].

Correlations between biological expression and response to chemotherapy (CR, PR, progression, and no response) at the primary site and neck nodes were performed using the χ^2 test, unless the number of observations was inadequate, in which case a Fisher's exact test was used [26]. Overall survival (OS) was calculated as the time lapse between registration into the study and death from any cause. Time to treatment failure (TTF) was defined as the time lapse between the date of registration into the study and the date of the first documented evidence of progressive disease. Disease-free survival (DFS) was defined as the interval between diagnosis and local, or systemic, relapse or death, whichever occurred first. These three parameters were estimated using Kaplan–Meier procedures [27]. The following variables were included in the univariate analyses: Karnofsky PS status, age, weight loss, T stage, N stage, primary site, resectability, histology grade, p53 and EGFR values. Log-rank tests for significance in predicting response to chemotherapy, OS, DFS and TTF were performed for all of these variables. Each pre-treatment potential prognostic factor that was observed to be significant in predicting OS (i.e. $P < 0.05$ on the log-rank test) was then introduced into a multivariate analysis using Cox's proportional hazards model [28] with forward stepwise regression and with the significance threshold set at 0.05. Then, while including only those variables that remained significant in the multivariate model, each biological marker was added separately into the Cox's proportional hazards model and re-analysed in a forward stepwise regression with the threshold of significance set at 0.05. The biological markers were re-analysed in the same procedures to assess their value in predicting DFS and TTF. Median follow-up data (upto April 2003) was computed using the inverse Kaplan–Meier procedures [29]. Non-parametric data were analysed using the χ^2 test. If the number of observations was inadequate, the Fisher's exact test was used [26]. Differences were considered significant if the P value (two-sided) was <0.05.

3. Results

3.1. Population characteristics

Archival representative blocks from the primary tumours were available for 46 of the 70 patients included in this phase II study [5,22]. The reasons for unavailability of specimens in the remaining 24 patients were the following: diagnosis outside of our institution, with no available tissue in the archival bank (9 patients), and insufficient sample for the immunohistochemical analysis (15 patients). Table 1 displays the characteristics of the group of patients studied ($n = 46$). No significant differences at baseline were observed between those from whom tissue samples were available for analysis compared with those from whom these were unavailable (data not shown).

Immunohistochemistry staining of p53 and EGFR were performed in all 46 cases. The p53-positive phenotype, defined as $\geqslant 50\%$ of tumour cell showing nuclear immunoreactivity, was observed in 26 (57%) of 46 cases.

Table 1
Patients' characteristics

Characteristics	Number (%) Total $n = 46$
Age (years) (median, range)	56.5 (43–73)
Males	45 (98)
Karnofsky PS	
90–100	40 (87)
80	6 (13)
Histology grade	
Well-differentiated	15 (33)
Moderately differentiated	20 (43)
Undifferentiated	8 (17)
Unknown	3 (7)
p53 status	
$\geqslant 50\%$	26 (57)
$<50\%$	20 (43)
EGFR status	
1+	18 (39)
2+	9 (20)
3+	19 (41)
Tumour size	
T1–T2	6 (13)
T3–T4	38 (83)
Tx	2 (4)
Nodal status	
N0–N1	17 (37)
N2–N3	29 (63)
Primary tumour site	
Oral cavity	8 (17)
Primary tumour site	
Oropharynx	14 (30)
Hypopharynx	13 (28)
Larynx	9 (20)
Unknown	2 (4)
Tumour resectability	
Resectable	15 (33)
Non-resectable	31 (67)

PS, performance status; EGFR, epidermial growth factor receptor.

Table 2
Clinical data segregated with respect to immuno-phenotype profile

Characteristics	EGFR+ ($n = 18$)	EGFR++ ($n = 9$)	EGFR+++ ($n = 19$)
Median age (years)	58	55	55
Karnofsky PS			
100	7	1	5
80–90	11	8	14
p53 status			
$<50\%$ (negative)	7	6	7
$\geqslant 50\%$ (positive)	11	3	12
Tumour size			
T1	–	–	1
T2	3	1	1
T3	3	2	9
T4	11	5	8
Tx	1	1	–
Nodal status			
N0	2	1	2
N1	5	2	5
N2	7	3	8
N3	4	3	4
Tumour site			
Oral cavity	3	–	5
Oropharynx	4	3	7
Hypopharynx	6	3	4
Larynx	4	2	3
Unknown	1	1	–
Resectable tumours			
	6	4	5

All the samples showed a positive staining for EGFR, with 28 specimens (61%) being 2+ or more. Although the size of our study sample limits the power of such estimations, there were no statistically significant differences in the baseline characteristics among patients with different EGFR staining scores. There were no significant differences between p53-negative and p53-positive patients with respect to any of the parameters measured (Table 2) and neither were there any significant correlations with the two markers.

3.2. Response to induction chemotherapy

Univariate analyses were conducted to assess relationships between clinical characteristics and the two biological markers in relation to response to induction therapy. The response at the primary site was considered separately because not all patients were N-positive (N0 = 8 patients) and also because among those patients who have node involvement the response to chemotherapy often differs (i.e. a PR at one site and a CR at the other). CR was observed at the primary site in 34 patients (74%, 95% Confidence Interval (CI): 64–85), and at the neck in 28 patients (61%, 95% CI: 49–75). PR at the primary localisation was achieved in 7 patients (15%, 95% CI: 2–28) and at the neck in 10 patients (22%, 95% CI: 5–39). The overall response rate was 88% (95% CI: 62–97).

Low levels of p53, defined as <50% of cells in the sample showing nuclear immunoreactivity, were observed in 83% of patients achieving a CR and in 50% of non-responders. This difference was statistically significant ($P < 0.05$), but was non-significant when the CRs and PRs were considered together.

Patients with tumours located in the hypopharynx and larynx achieved CRs or PRs more often than those with tumours located in the oral cavity and oropharynx ($P < 0.05$). EGFR expression did not correlate with response in this group of patients ($P > 0.05$).

3.3. Disease-free survival and time to treatment failure

Univariate analysis was used to compare the EGFR immunohistochemistry staining (1+ *versus* 2+ *versus* 3+) with treatment outcome in terms of DFS and

TTF. Median TTF was 21.2 and 23.8 months (3+ versus 2+, respectively), while all patients with an EGFR of 1+ were alive and without evidence of disease at the time of the analysis (median = 60 months of follow-up; $P < 0.05$).

No differences were observed between p53-positive and p53-negative patients in terms of DFS and TTF. However, patients who were p53-positive and had moderate-to-high EGFR staining (2+ and 3+) had a TTF of 14.9 months, whereas the patients who did not have these two features had longer median TTFs. Significant differences were observed in these two sub-groups of patients in terms of DFS (21.2 months versus 55.7 months, $P < 0.05$). Tumours located in the oral cavity showed poorer DFS and TTF when compared with other tumour locations.

The rest of the variables in the univariate analysis failed to reach statistical significance.

On multivariate analysis, tumour location within the oral cavity was an independent prognostic factor of a shorter DFS. Levels of p53 expression in combination with moderate-to-high EGFR staining were also of independent prognostic value with respect to DFS (Fig. 1) and TTP.

3.4. Overall survival

The median OS for the entire group was 30.8 months (range 10.5–51.2 months). On univariate analysis, EGFR expression was strongly associated with a shorter OS; the median OS being 20 months for patients with 2+/3+ EGFR expression, whereas all of the patients with 1+ are currently alive (median follow-up of 60 months; $P < 0.05$). Tumour location in the oral cavity was also associated with a poorer prognosis in terms of a shorter OS.

The rest of the variables included in the univariate and multivariate analyses did not show prognostic significance. The variables included were p53 status, histological grade, performance status, TNM stage, and resectability.

The presence, in combination, of positive p53 staining and 2+ or 3+ of EGFR staining were associated with shorter OS times (21.2 versus 55.7 months, $P < 0.005$).

In the multivariate analyses employing the Cox model, oral cavity location and EGFR expression had statistically significant independent prognostic value in our group of patients (Fig. 2).

4. Discussion

The specific aim of this study was to determine whether the expression of p53 and EGFR were indicators of response to induction chemotherapy and clinical outcome in patients with advanced HNSCC. Although most patients with advanced head and neck carcinoma have a poor prognosis, a small subset of patients respond favourably to induction chemotherapy. CR to induction chemotherapy has been shown to be associated with better survival rates [5]. To date, only TNM stage seems to predict clinical outcome [6]. However, this system often fails to predict individual response to treatment. Hence, identification of other predictors of tumour response would be invaluable in defining a more

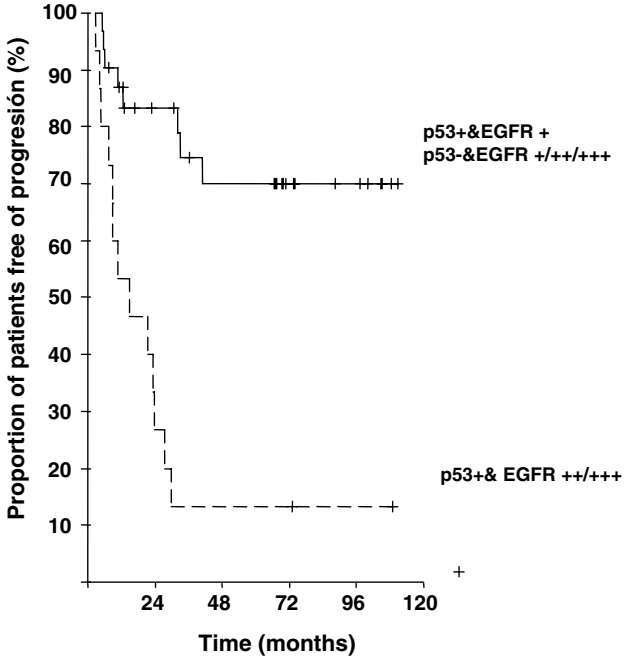

Fig. 1. Relationship between disease-free survival and expression of epidermal growth factor receptor (EGFR) and p53.

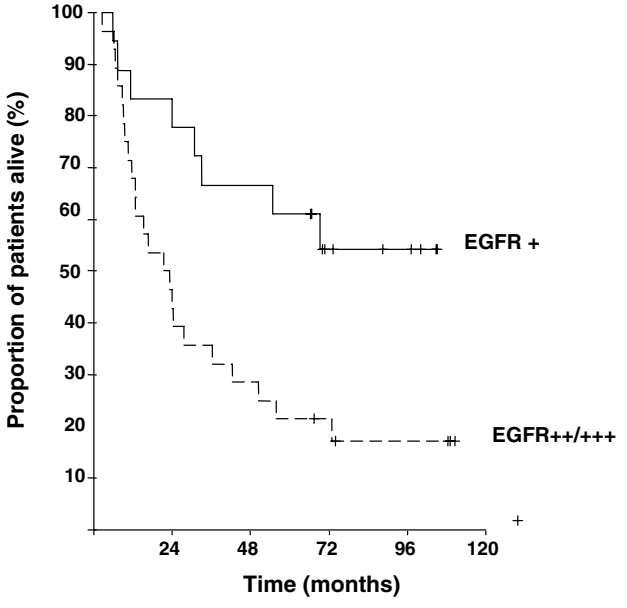

Fig. 2. Relationship between overall survival and EGFR expression.

rational selection of patients for induction chemotherapy.

Our study population were a group which was homogeneously treated with induction chemotherapy based on a cisplatin, 5-flourouracil, and paclitaxel regimen [22]. In our series of patients, >50% had a positive nuclear staining for p53. The cut-off point employed to define positive staining for p53 was $\geqslant 50\%$ cells staining positive. Such a high cut-off value decreases the number of false-positive results included in the final analysis. Our analysis indicated a strong correlation between nuclear p53 staining and a poor prognosis, in terms of response to induction chemotherapy. These results are concordant with other studies previously reported [30]. However, we found that p53 status constituted a prognostic factor in terms of DFS and OS only when this expression was combined with overexpression of EGFR. For response to induction chemotherapy, the positivity of p53 was an adverse prognostic factor only in those patients who achieved CR. When CRs and PRs were combined, the p53 status was not an independent prognostic variable. We had evaluated the prognotic significance of p53 previously in Ref. [24], in a series of patients treated with cisplatin and 5-fluorouracil induction therapy and we had observed a trend, albeit statistically non-significant, towards lower response rates in patients who were p53-positive. In a recent study [31], no correlation between p53 overexpression and response to a carboplatin-paclitaxel regimen had been noted and, in this respect, discordant results have been reported in the literature [32–35]. Clearly, the true predictive value of p53 overexpression in head and neck carcinoma has yet to be defined and the usefulness of this immunohistochemical staining as a prognostic and/or predictive factor has yet to be determined [36].

All our patients had a positive staining for EGFR and in 61% the staining intensity was moderate to high. We did not observe any difference in baseline characteristics in our group of patients with respect to EGFR status. Over a decade ago, Santini and colleagues reported, in a study in which 32 of 70 patients were treated with cisplatin-5-fluorouracil induction chemotherapy and compared with a control arm, a significant correlation between EGFR levels and TNM stage, as well as a high probability of achieving CR to chemotherapy [18]. Grandis and colleagues demonstrated that EGFR overexpression was independently prognostic for both local control and OS in a group of 91 patients treated with surgical resection and post-operative radiation therapy [21]. They concluded that EGFR overexpression contributed to a more aggressive phenotype resulting in a greater risk of death from the disease. Other authors have reported this poorer prognosis in patients overexpressing EGFR and receiving a variety of treatments [16,19,21]. We did not find any significant correlation between overexpression of EGFR and response to induction chemotherapy. This might be due to the lack of relationship between response to taxanes and the EGFR signalling pathway. We observed that overexpression of EGFR was associated with a lower DFS and OS; this difference being higher when those tumours with low-to-moderate expression were compared with those with a high expression of EGFR. This finding is of considerable importance because patients treated with the PPF regimen achieved high response rates, and included patients with high expression of EGFR. However, this overexpression was related to OS rates. Our results are concordant with those previously reported [19,21]. Elevated levels of EGFR have been related with increased tumour size and advanced tumour stage [37]. In our study, we did not observe these relationships, probably due to the limited sample size and/or the advanced disease stage of our group of patients.

In our study, 87% of the patients had a PS of $\geqslant 90\%$ on the Karnofsky scale. Our patients had advanced stages of HNSCC, with 83% being T3–4, 63% being N2–3, and 67% being non-resectable. Only tumour location at the oral cavity and oropharynx were independent factors of a poorer prognosis, with no significant relationships with any of the other variables including tumour size, nodal stage, and histological grade or performance status being observed. Several studies have analysed the prognostic importance of TNM stage and histological grade in patients treated with induction chemotherapy [38–40]. However, most of these studies were conducted with chemotherapy regimens that are not in use today, and in very heterogeneous groups of patients. In our study, all of the 46 patients whose tumour samples were available for the immunohistochemical analysis had been treated with the same regimen in a phase II clinical trial [22]. To the best of our knowledge, this is the first study of its kind conducted in a homogenously treated group.

In our study, location in the oral cavity and moderate-to-high EGFR expression appeared to have independent prognostic value on multivariate analysis. These results are concordant with previously reported studies [21,41,42]. The p53 status appears to be a determinant of the chemo-sensitivity in advanced HNSCC patients. The presence, in the tumour, of cells staining positive for p53 and moderate-to-high staining for EGFR was associated with a shorter DFS and TTF and probably reflects, a more aggressive tumour phenotype. Although the small number of patients limits the value of our findings, these results provide a sufficient basis to warrant further studies to determine prognostic and predictive value of p53, EGFR, and other molecular markers in patients with advanced HNSCC.

Conflict of interest statement

None declared.

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