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Original Paper

Amplification of *ERBB* Oncogenes in Squamous Cell Carcinomas of the Head and Neck

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The activation of *ERBB* oncogenes has been described in various human tumours, including squamous cell carcinomas of the head and neck (SCCHN), and, in some of them, it has been correlated with a poor prognosis. Tissue samples from 59 patients with SCCHN were studied. After DNA extraction, the *ERBB1*, *ERBB2* and *ERBB3* copy number in tumour samples was estimated with the polymerase chain reaction (PCR) method. The PCR products were analysed by agarose gel electrophoresis and quantified by image analysis techniques. 9 (15%) cases presented with *ERBB1* amplification, which was correlated with lymph node involvement ($P = 0.04$), poorly differentiated tumours ($P = 0.03$) and a hypopharyngeal primary site ($P = 0.035$). No correlation among amplification status, recurrence, metastases and survival was observed, although this may be due to the small number of patients in the amplified group. None of the 59 cases presented amplification of *ERBB2* and *ERBB3* oncogenes.

Key words: head and neck squamous cell carcinoma, DNA analysis, polymerase chain reaction, amplification, epidermal growth factor receptor, *ERBB2*, *ERBB3*

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INTRODUCTION

THE *ERBB* (epidermal growth factor receptor-related) family of growth factor receptors is increasingly recognised as being important in the control of the normal cell proliferation and in the pathogenesis of human cancer [1]. The family comprises four closely related members: the epidermal growth factor receptor (EGFR, *ERBB1*), *ERBB2/NEU*, *ERBB3* and the recently described *ERBB4*. The *ERBB1* gene has been found amplified and/or overexpressed in various human tumours [2–4], including squamous cell carcinomas of the head and neck (SCCHN) [5–12]. Overexpression of the *ERBB1* has been consistently found in SCCHN, and it was suggested that this might be a poor prognostic factor [12]. However, amplification of *ERBB1* has not been shown to be a consistent feature of SCCHN [13, 14], and no previous studies have correlated *ERBB1* amplification with long-term survival rates. *ERBB2* has often been found amplified in adenocarcinomas of various origins [4, 15, 16], but there is no evidence for amplification of *ERBB2* in SCCHN [6–9, 13] or overexpression of *ERBB2* RNA transcripts [17, 18]. However, in two studies of *ERBB2* oncoprotein expression in SCCHN, 46 and 60% of the specimens were positive for *ERBB2* staining [19, 20]. The third member of this gene family, *ERBB3*, has been found to be expressed in normal epithelial cells throughout the gastrointestinal tract and in tumours arising at these sites [21]. Overexpression of *ERBB3* has been described in breast carcinomas [22] and in head and neck cancer cell lines [23], but no gene amplification of *ERBB3* was noted in these studies.

The aim of this study was to determine the frequency of *ERBB* family amplification in SCCHN by differential polymerase chain reaction (PCR) and hence to assess whether *ERBB* amplification correlates with clinicopathological data and prognosis. Differential PCR [24, 25] is a rapid, simple and sensitive method for semiquantitatively determining the number of gene copies, that only requires small amounts of

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Table 1. Oligonucleotide primers used in differential PCR

Gene	Oligonucleotide primers	Size (bp)*
ERBB1 [4]	5′-ACAGCCATGCCCGCATTAGCTCTAA-3′ 5′-GGAATGCAACTTCCCAAAATGTGCC-3′	109
ERBB2 [24]	5′-CCTCTGACGTCCATCATCTC-3′ 5′-ATCTTCTGCTGCCGTCGCTT-3′	98
ERBB3 [26]	5′-GTTGATGACCTTCGGGGC-3′ 5′-GCAGATCTGGGGCTGTGC-3′	83
IFNG [24]	5′-TCTTTTCTTTCCCGATAGGT-3′ 5′-CTGGGATGCTCTTCGACCTC-3′	150
β-actin [4]	5′-TATCCAGGCTGTGCTATCCCTGTAC-3′ 5′-CTTGATGAGGTAGTCAGTCAGGTCC-3′	169

* Length of the PCR amplified fragment.

DNA, and should facilitate these studies in clinical medicine.

MATERIALS AND METHODS

Tumour specimens

Between April 1991 and February 1993, tissue samples of 59 consecutive primary SCCHN were obtained from patients undergoing surgical resection of their tumour. Whenever possible, adjacent, apparently normal tissue was also obtained. Tissue specimens were frozen immediately in liquid nitrogen and stored at −70°C until DNA extraction. All patients included in our study had a single primary tumour, none had undergone treatment prior to surgery, and clear surgical margins were obtained in all cases. As a general rule, the patients with histologically N2 or N3 neck lesions received a course of postoperative radiotherapy (55 Gy), which was also administered in N0-N1 neck lesions with locally advanced stages (T4). All patients except one were male, with a mean age of 58.3 years (range 38–85 years). The primary sites of the tumours according to patient distribution were: oral cavity ($n = 4$), oropharynx ($n = 18$), supraglottic larynx ($n = 9$), glottic larynx ($n = 7$) and hypopharynx ($n = 21$).

The stage of disease was determined according to the TNM system of the UICC (4th edition). One patient had stage I disease, four had stage II, 20 had stage III and 34 had stage IV.

DNA isolation and PCR analysis

The tissues were minced, homogenised and digested twice with 100 μg proteinase K (Boehringer Mannheim, Mannheim, Germany) per milliliter of digestive buffer (200 mM Tris–HCl pH 8.0, 25 mM EDTA, 100 mM NaCl and 0.2% sodium dodecyl sulphate) for 8 h at 37°C. High-molecular weight DNA was then isolated by standard phenol-chloroform extraction, precipitated in ethanol, dissolved in 10 mM Tris pH 8.0, 1 mM EDTA and stored at −20°C. DNA concentration was measured by absorbance at 260 nm.

The PCR mixtures contained 1 μg of target DNA, 10 mM Tris–HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.005% Tween-20, 0.005% NP-40, 0.01% gelatin, 0.2 mM of each dNTP, 1 μM of each primer and 1 U of Taq polymerase (Boehringer Mannheim, Mannheim, Germany) in 50 μl total volume with a 50 μl mineral oil overlay. The PCR cycles included 1 min at each temperature (94°C, 56°C and 72°C), for a total of 35 cycles, and a final cycle at 72°C for 7 min. Two different sets of primers, one for the target gene (*ERBB1, ERBB2* or *ERBB3*) and the other for the control gene, were present simultaneously in the reaction vessel, as previously described [24, 25]. Firstly, we used as the control gene the gene for interferon-gamma (*IFNG*), which was the control gene used in the first description of the technique [24]. Positives from this reaction were verified using a second reference gene located on the same chromosome as the target gene (e.g. the gene for β-actin for the *ERBB1*). The primers were synthesised with an automated DNA synthesiser (Applied Biosystems, Inc., Foster City, California, U.S.A.). The sequences of the primers used and the predicted PCR products are listed in Table 1 [4, 24, 26].

Electrophoresis and quantitation of PCR products

After PCR, 10 μl of each sample were electrophoresed directly on gels containing 3% NuSieve agarose (FMC, Rockland, Maine, U.S.A.) and 1% molecular biology grade agarose (Promega, Madison, Wisconsin, U.S.A.) for 1.5 h at 65 V in 40 mM Tris–acetate, 2 mM EDTA buffer. The gels were stained with ethidium bromide, and the ultraviolet-illuminated gels were photographed with Polaroid 667 film. The bands were quantitated by computerised densitometric analysis techniques (Bio Image, Millipore, Ann Arbor, Michigan, U.S.A.) and the *ERBB1, ERBB2* or *ERBB3/IFNG*, and *ERBB1*/β-actin ratios were determined.

Statistical analysis

Statistical analysis was performed using Chi-squared tests with Yates' correction where appropriate. Logistic regression models were used to examine the independent effects of variables related to *ERBB* amplification in univariate analyses. Survival curves were calculated using the Kaplan–Meier product limit estimate [27]. The event analysed was the death of a patient related to tumour progression; all other survival times were treated as right-censored. Differences between survival times were analysed by the log-rank method [28]. Patients were observed for at least 26 months (mean follow-up 41 months).

RESULTS

A total of 59 samples from patients with SCCHN were studied. Clinicopathological data from the patients are shown in Table 2. Each sample was investigated for *ERBB1, ERBB2* and *ERBB3* amplification by differential PCR using the gene for *IFNG* as a control. Samples of DNA obtained from normal tissues were used as controls, showing *ERBB1/IFNG* ratios of 1.1 ± 0.1 (mean ± S.D.), *ERBB2/IFNG* ratios of 0.9 ± 0.1 and *ERBB3/IFNG* ratios

Table 2. Clinicopathological data of patients and ERBB1 *amplification*

Case no.	Age (years)	Site	T-stage	N-stage	Pathological grade	ERBB1 amplification	Status (months)
1	68	Oropharynx	4	3	Mod.	–	DOC(26)
2	68	Larynx	4	0	Poor	–	D (3)
3	43	Hypopharynx	4	2	Poor	+	D (10)
4	78	Hypopharynx	4	2	Poor	+	DOC (8)
5	63	Oropharynx	4	2	Mod.	+	D (15)
6	46	Oropharynx	3	2	Mod.	–	DFS (50)
7	52	Hypopharynx	2	0	Poor	–	D (15)
8	60	Hypopharynx	2	0	Well	–	DFS (50)
9	70	Oropharynx	2	2	Well	–	DOC (3)
10	70	Larynx	4	2	Well	–	DFS (49)
11	68	Hypopharynx	4	0	Well	–	D (12)
12	44	Oropharynx	3	1	Mod.	–	D (11)
13	67	Oropharynx	3	1	Mod.	–	DOC (27)
14	65	Larynx	2	1	Well	+	D (16)
15	54	Oropharynx	4	1	Well	–	D (9)
16	54	Oral cavity	4	2	Well	–	D (3)
17	51	Hypopharynx	3	2	Well	–	D (34)
18	61	Hypopharynx	4	2	Well	–	D (22)
19	47	Hypopharynx	3	0	Well	+	D (10)
20	43	Oropharynx	2	2	Poor	–	D (30)
21	54	Hypopharynx	1	1	Poor	+	DFS (46)
22	38	Oral cavity	4	2	Well	+	DFS (46)
23	53	Oropharynx	3	2	Well	–	D (6)
24	45	Larynx	1	0	Well	–	DFS (44)
25	62	Hypopharynx	4	1	Well	+	DOC (3)
26	71	Larynx	2	3	Mod.	–	DOC (10)
27	63	Oropharynx	3	0	Well	–	DOC (1)
28	63	Oropharynx	4	3	Well	–	D (16)
29	56	Larynx	2	0	Well	–	DFS (43)
30	53	Hypopharynx	2	1	Well		DFS (43)
31	61	Larynx	2	1	Mod.	–	DFS (43)
32	76	Hypopharynx	4	0	Mod.	–	DFS (42)
33	63	Oropharynx	4	2	Mod.	–	D (7)
34	59	Larynx	2	1	Well	–	DOC (28)
35	49	Oropharynx	2	2	Mod.	–	D (19)
36	51	Oropharynx	4	0	Poor	–	DOC (30)
37	42	Oral cavity	4	2	Mod.	–	D (18)
38	64	Larynx	3	1	Well	–	DFS (42)
39	62	Larynx	2	0	Well	–	D (18)
40	57	Larynx	3	0	Well	–	DFS (34)
41	56	Hypopharynx	3	0	Mod.	+	D (34)
42	62	Oropharynx	3	2	Well	–	D (14)
43	59	Larynx	3	0	Well	–	DFS (34)
44	48	Hypopharynx	2	3	Mod.	–	DFS (33)
45	63	Larynx	3	2	Mod.	–	DOC (2)
46	42	Oral cavity	2	1	Well	–	D (9)
47	62	Hypopharynx	3	0	Mod.	–	DOC (26)
48	57	Oropharynx	4	2	Mod.	–	DFS (33)
49	53	Oropharynx	4	2	Well	–	D (7)
50	59	Hypopharynx	3	2	Well	–	D (10)
51	57	Larynx	3	0	Well	–	DFS (31)
52	49	Hypopharynx	4	0	Mod.	–	D (16)
53	54	Hypopharynx	2	3	Mod.	–	D (7)
54	71	Oropharynx	3	0	Well	–	D (16)
55	67	Hypopharynx	3	0	Well	–	DOC (27)
56	85	Larynx	4	0	Well	–	DFS (30)
57	64	Oropharynx	3	0	Well	–	DFS (30)
58	61	Larynx	3	2	Well	–	D (7)
59	60	Hypopharynx	3	1	Well	–	D (9)

Well, well differentiated; Mod., moderately differentiated; Poor, poorly differentiated; D, dead with tumour; DOC, died from other causes without evidence of disease; DFS, disease-free survival. *ERBB1* amplification: +, amplified; –, non-amplified.

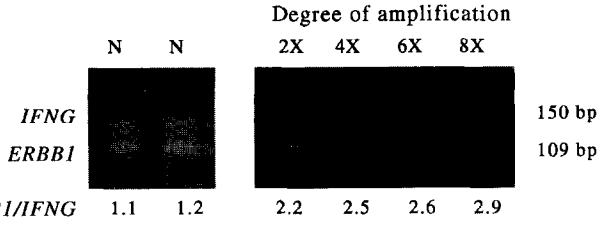

Figure 1. Electrophoretic separations of PCR fragments from normal tissues (N), and positive controls mimicking 2-, 4-, 6- and 8-fold amplification of the *ERBB1* gene. The PCR conditions and the quantitation of the PCR products are as described in the text.

of 1 ± 0.1 (mean $\pm$ standard deviations obtained from 10 different samples). As positive controls, a mixture of DNA from normal tissue and increasing amounts of a previously PCR-amplified sequence of the target gene (*ERBB1, ERBB2* or *ERBB3*), mimicking different degrees of amplification, was used as the template in the PCR reaction. The data showed that there was an increase in the PCR product from the target gene, compared with the PCR product from the control gene, as the target gene copy number used as the template was increased (Figure 1). Similar results were obtained when we used either 25, 30, 35, 40 or 45 PCR cycles (data not shown). Thus, differential PCR detects gene amplification, and the results were independent of the number of PCR cycles used, as previously described [24, 25]. Experimentally, gene amplification was defined as at least a 2-fold increase in the *ERBB1/IFNG, ERBB2/IFNG* and *ERBB3/IFNG* ratios, relative to the ratios obtained with normal tissue DNA. Because of the semiquantitative nature of the differential PCR, the ratios obtained were not converted to a score of amplification, the results being expressed as amplification or no amplification of the *ERBB* genes.

Nine (15%) of the 59 samples demonstrated *ERBB1* amplification. The *ERBB1/IFNG* ratios obtained in these cases are shown in Figure 2a. None of the cases considered non-amplified presented a *ERBB1/IFNG* ratio higher than 1.5 (mean $\pm$ S.D., 1.1 ± 0.13). The positive cases were verified using the gene for β-actin (which is located in the same chromosome as *ERBB1*) as reference. In this manner, false positive cases due to chromosomal duplications can be effectively eliminated. Normal and positive controls were obtained as described above, and amplification was also defined as at least a 2-fold increase in the *ERBB1*/β-actin ratio relative to the ratios obtained with normal tissue DNA. All the positive cases were confirmed and, in consequence, were considered true amplifications (Figure 2b). Normal adjacent mucosa tissue was available in five of these cases and none contained *ERBB1* amplification.

No patient presented amplification of *ERBB2* (mean $\pm$ S.D. *ERBB2/IFNG* ratio, 0.8 ± 0.05) or *ERBB3* (mean $\pm$ S.D. *ERBB3/IFNG* ratio, 1.1 ± 0.1) genes (Figure 3).

Table 3 presents the correlation of *ERBB1* amplification with clinical stage, pathological grading, primary site and recurrence. Amplified tumours were found at each primary head and neck anatomical site except the glottis, with a significantly higher frequency of amplification (6 of 21 cases, 29%) in those with a hypopharyngeal primary site ($P = 0.035$). Although five (56%) of the nine cases with *ERBB1* amplication were observed in T4-stage tumours, there was no statistically significant association between T-stage and *ERBB1* amplification ($P = 0.32$). Of the nine *ERBB1* amplified cases, eight (89%) had cervical lymph node disease, whereas only 31 (62%) of the 50 non-amplified had cervical lymph node metastases ($P = 0.04$). None of the six T4-stage tumours without lymph node metastases presented *ERBB1* amplification, whereas five (33%) of the 15 with lymph node disease showed *ERBB1* amplification ($P = 0.02$). Moreover, one T1-stage tumour with cervical lymph node metastases also presented *ERBB1* amplification. Therefore, the relationship between *ERBB1* amplification and nodal involvement seems to be independent of T-stage. However, using a logistic regression model to examine the independent effect of *ERBB1* and T-stage in nodal metastases, none of these variables was statistically significant. None of the patients with either stage I or II disease had *ERBB1* amplification, whereas 3 (15%) of the 20 with stage

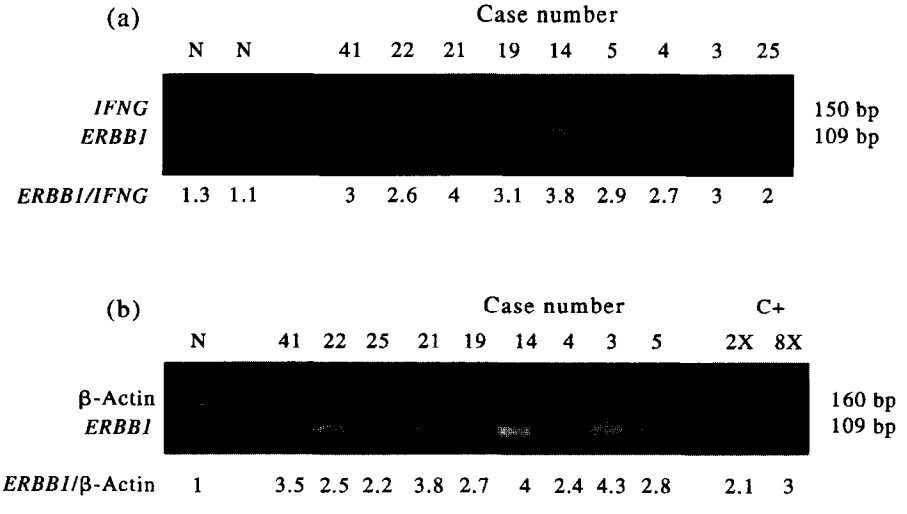

Figure 2. Differential PCR analysis of the nine tumour samples with *ERBB1* amplification: (a) using *IFNG* as a reference gene, and (b) using β-actin as a reference gene. The PCR products were separated by agarose gel electrophoresis and quantified as explained in the text. N indicates the controls with normal tissues; C +, the positive controls mimicking 2-fold and 8-fold amplification of the *ERBB1* gene; and bp, base pairs.

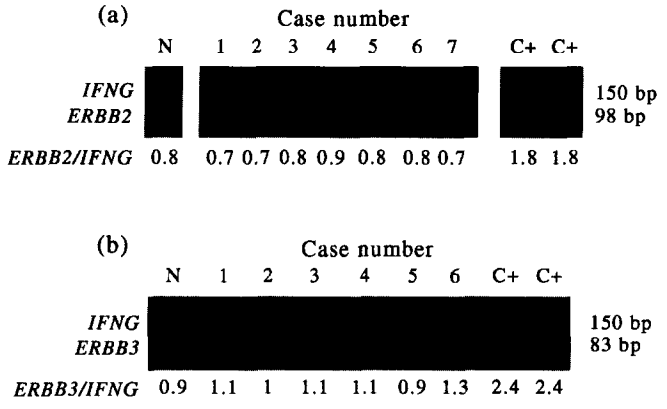

Figure 3. Differential PCR analysis of *ERBB2* (a) and *ERBB3* (b) gene amplification from various tumour samples. None of the samples showed gene amplification. The PCR products were separated by agarose gel electrophoresis and quantified as explained in the text. N indicates controls with normal tissues; C +, the positive controls mimicking 2-fold amplification; and bp, base pairs.

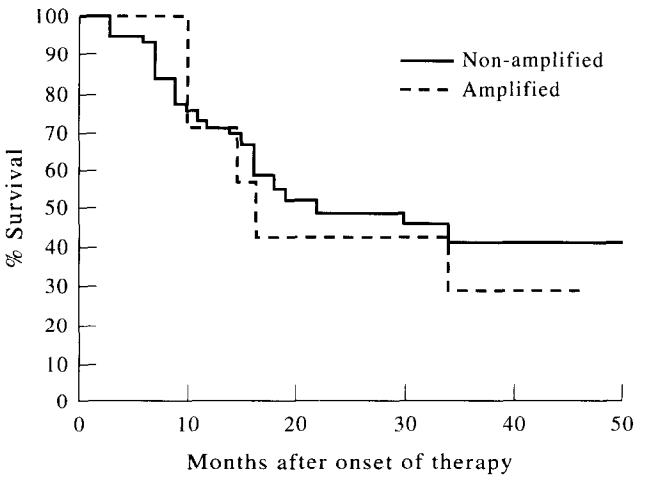

Figure 4. Disease-free survival curves of the patients with and without *ERBB1* gene amplification. Survival curves were drawn-up using the Kaplan–Meier product limit estimates.

III disease and 6 (18%) of the 34 with stage IV disease had *ERBB1* amplification, but this did not reach statistical significance, possibly because of the small number of cases in stages I–II. Three (43%) of seven poorly differentiated tumours had *ERBB1* amplification, compared with 2 (11%) of the 19 moderately differentiated tumours and 4 (12%) of the 33 well differentiated tumours ($P = 0.09$). Grouping the well and moderately differentiated tumours together, the results were significantly different ($P = 0.03$).

6 patients who died from other causes in the first 2 years after initial treatment were excluded from the recurrence analysis because of insufficient follow-up (two were in the amplified group). Five of the seven (71%) amplified cases had local recurrence or distant metastases, compared with 29/46 (63%) in the non-amplified group, which was not statistically significant ($P = 0.61$). Similarly, there were no statistically significant differences in survival between patients with and without *ERBB1* amplification (Figure 4).

DISCUSSION

Despite the structural similarities between the members of the *ERBB* family of growth factor receptors, there are marked dissimilarities in the activation of their genes in SCCHN. *ERBB1* gene amplification has been frequently found in SCCHN [5–10], but, to the best of our knowledge, SCCHN with *ERBB2* or *ERBB3* amplification are yet to be reported [6–9, 13, 23].

In our study, nine (15%) of 59 primary SCCHN presented amplification of *ERBB1*. This is in agreement with previous studies, in which the reported rates range from 7 to 25% [5–10]. There are also studies in which *ERBB1* amplification has not been found, but the number of cases studied was small [13, 14]. Our results show that *ERBB1*

Table 3. ERBB1 amplification by stage, pathological findings, site and relapse

Parameter	No. of cases	Amplified no. (%)	*P* value*
T-stage			
T1-T3	38	4(11)	
T4	21	5(24)	0.32
N-stage			
N0	20	1(5)	
N+	39	8(21)	0.04
Pathological grade			
Well differentiated	33	4(12)	
Moderately differentiated	19	2(11)	
Poorly differentiated	7	3(43)	0.09
Site			
Oral cavity	4	1(25)	
Oropharynx	18	1(6)	
Supraglottic	9	1(11)	
Glottis	7	0(0)	
Hypopharynx	21	6(29)	0.035
Recurrence†			
No recurrence	19	2(11)	
Loco-regional recurrence or distant metastases	34	5(15)	0.61

* Chi-squared test. † 6 patients who died from other causes were excluded due to insufficient follow-up.

amplification correlates with cervical lymph node metastases and that this association seems to be independent of T-stage. This finding, however, should be interpreted with caution because of the small number of patients with amplification, but it agrees with the results of Irish and Bernstein [5].

As for the link between histological differentiation and *ERBB1* amplification in SCCHN, the data are conflicting. Some authors have shown a correlation between *ERBB1* amplification and poorly differentiated tumours [9], others have reported this correlation with well differentiated tumours [6], and, finally, others have found no relationship at all [7, 8]. We found a significant association of *ERBB1* amplification with poorly differentiated tumours, although amplification was also found in well and moderately differentiated tumours.

Previous studies have demonstrated the value of *ERBB1* overexpression as an indicator of poor prognosis in SCCHN [12]. *ERBB1* amplification has also been suggested as a marker for a poorer prognosis in these tumours, as it has been linked to more advanced clinical disease [5]. However, no previous studies have correlated *ERBB1* amplification with long-term survival rates. In our study, despite the association with nodal involvement and poorly differentiated tumours, there were no statistically significant differences in 3-year survival between patients with and without *ERBB1* amplification. Since the most single important prognostic factor that has been found correlated with survival in SCCHN is the presence of positive lymph nodes, the lack of correlation may be due to the small number of patients in earlier stages I and II of the disease.

ERBB1 amplification was associated in the present study with a hypopharyngeal primary site. This contrasts with published data in SCCHN in which no correlation between *ERBB1* amplification and site of the tumour was found [6, 7]. However, a similar association has recently been described for chromosome 11q13 amplification [29]. These results cannot be attributed to a more advanced nodal stage for hypopharyngeal carcinomas (62% had lymph node metastases) than those with a different location (68% had cervical lymph node metastases), and may reflect a particular feature of the hypopharyngeal site.

In agreement with previous studies [6–9, 13, 23], we could not demonstrate either *ERBB2* or *ERBB3* gene amplification in our tumour samples. Alterations of the *ERBB2* gene or its expression have been observed in adenocarcinomas and carcinomas of glandular tissue [15, 16], but, up to now, never in squamous cell carcinomas [6–9, 13, 17, 30]. Therefore, the lack of *ERBB2* gene amplification in SCCHN may be characteristic of this tumour type and suggests differences in aetiology between adenocarcinoma and squamous cell carcinoma. More limited information is available on the involvement and role of *ERBB3* gene amplification in the development and prognosis of human tumours. No previous work has reported *ERBB3* amplification in the tumours studied [22, 23], which suggests that amplification is not a common mechanism of activation of this gene.

In summary, our study confirms previous reports that *ERBB1*, but not *ERBB2* or *ERBB3*, amplification can be present in primary human SCCHN. In addition, we have found a significant correlation between *ERBB1* amplification and cervical lymph node metastases. However, this was not correlated with a worse prognosis for the patients with *ERBB1* amplification. Caution should be taken in this regard given the small number of patients with amplification. Therefore, the prognostic value of this marker should be investigated in wider series of patients, especially in earlier stages of the disease.

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