Pergamon

European Journal of Cancer, Vol. 33, No. 13, pp. 2203–2210, 1997

Printed in Great Britain
0959-8049/97 $17.00+0.00

PII: S0959-8049(97)00198-6

Original Paper

Amplification of 11q13 DNA Markers in Head and Neck Squamous Cell Carcinomas: Correlation with Clinical Outcome

D. Muller,[1] R. Millon,[1] M. Velten,[2] G. Bronner,[4] G. Jung,[3] A. Engelmann,[5] H. Flesch,[4] M. Eber,[1] G. Methlin[1] and J. Abecassis[1]

[1]Department of Tumour Biology; [2]Department of Bio-Statistics; [3]Department of Radiotherapy, Centre Paul Strauss, 3 rue de la Porte de l'Hopital; [4]Clinique Saint Barbe; and [5]Laboratory of Pathology, 29 rue Oberlin, 67085 Strasbourg Cedex, France

This study was performed on 282 patients with primary head and neck squamous cell carcinomas to evaluate the prognostic importance of 11q13 amplification. Amplification of the 11q13 DNA markers, *HST-1/FGF-4* and *BCL-1*, evaluated by Southern and slot blot hybridisation, was detected in 52% of tumours. 11q13 amplification was associated with tumour site since this alteration occurred in 76% of tumours arising in the hypopharynx, versus 40% in the other sites (P=0.0007). 11q13 amplification was also significantly related to the presence of involved neck lymph nodes (P=0.013). The relationship between 11q13 amplification and risk of progression was studied in two subgroups of head and neck cancer patients with regard to treatment modalities. The presence of 11q13 amplification in the tumour was not significantly associated with a shorter event-free survival (P=0.82) and crude survival (P=0.61) of the 201 patients treated by surgery and postoperative radiotherapy. Similarly, absence of a relationship was observed for the group of 79 patients treated by surgery alone. These results confirm that 11q13 amplification is a prominent event in head and neck squamous cell carcinoma, indicating that it may be a common genetic event in the development of these neoplasms, but is not a reliable prognostic marker. © **1997 Elsevier Science Ltd.**

Key words: head and neck, carcinoma, gene amplification, HST-1 gene, 11q13, prognostic
Eur J Cancer, Vol. 33, No. 13, pp. 2203–2210, 1997

INTRODUCTION

HEAD AND NECK cancer has a worldwide incidence of nearly 400 000 new cases per year. In Western countries, it accounts for approximately 2–4% of all carcinomas, with a high incidence of the disease in the eastern part of France (60/100 000 men per year) [1]. Head and neck carcinomas, 90% of which are of the squamous type, are characterised by local tumour aggressiveness, early recurrence and development of second primary cancer in 10–20% of cases. Advanced cancer remains a major therapeutic challenge despite the refinement of treatment strategy. Thus, the long-term prognosis for advanced head and neck cancer is still unsatisfactory, with an overall 5-year cure rate of 30–35%. The identification of reliable tumour markers that reflect tumour aggressiveness could be of relevance in order to characterise patients who will have a distinct outcome.

Among the molecular mechanisms which, potentially, could contribute to tumour development and/or progression of head and neck squamous cell carcinomas (HNSCC), alterations of the q13 region of chromosome 11 have been reported. The 11q13 region was originally described as rearranged in parathyroid adenomas [2], and translocated in B-cell neoplasms [3]. Moreover, amplification of the 11q13 region has been shown to occur, with varying frequency, in human solid malignancies i.e. 15% in breast cancer [4], 30–50% in HNSCC [5] or oesophagus [6], 30% in lung cancer [7]. Multiple genes reside in this amplicon, including the oncogenes *HST-1/FGF-4* and *INT-2/FGF-3* belonging to the fibroblast growth factor gene family, as well as the *cyclin D1/PRAD1* and the *BCL-1* locus breakpoint [8]. The usual coamplification of these genes strongly suggests their involvement in tumour progression. In fact, protein overexpression

Correspondence to D. Muller.
Received 20 Feb. 1997; revised and accepted 14 Apr. 1997.

is restricted to the *cyclin D1/PRAD1* gene [9] which encodes a cyclin D type protein playing a major role in G1 phase progression of the cell cycle [10].

We recently evaluated the amplification status of 11q13 DNA markers, namely *HST-1/FGF-4* oncogene and the *BCL-1* locus in a group of 178 HNSCCs [11]. A significant correlation was found between 11q13 amplification and usual prognostic factors such as lymph node involvement or advanced clinical stage. Clinically, the more relevant question is whether amplification of the 11q13 region may distinguish patients with a worse clinical outcome. Thus, in an attempt to assess the prognostic significance of 11q13 gene amplification, the study is now extended to 282 head and neck cancer patients treated in a single institution.

PATIENTS AND METHODS

Patients

282 consecutive unselected patients with operable HNSCC who had undergone primary tumour resection between February 1989 and June 1993, 178 of whom had been part of our previous study [11], were prospectively entered into the study. The mean age of the patients, including 257 males and 25 females, was 58 years (range 24–88 years). 34 patients presented synchronous primary head and neck tumours at the time of surgery; none presented distant metastasis. Patients were first treated with optimal tumour-reductive surgery. None of the patients had previously received radio- or chemotherapy. After primary surgery, 203 patients received adjuvant radiotherapy (30 of them had radiotherapy combined with chemotherapy) according to the TNM stage of their disease (Table 1). The median follow-up period of the whole series was 28 months with a maximum follow-up period of 68 months.

Table 1. *Characteristics of patients and treatments*

	Characteristic	Number	%
Patients	Total number	282	100
	Age		
	Mean (years)	58	
	Range (years)	24–88	
	Sex		
	M	257	91
	F	25	9
Tumours	Size (pathological)		
	pT1	49	17
	pT2	110	39
	pT3	90	32
	pT4	30	11
	PTx	3	1
	Nodal status (pathological)		
	pN0	65	25
	pN1	30	11
	pN2a,b,c	142	54
	pN3	26	10
	Unknown	19	—
	Differentiation grade		
	Well	94	34
	Moderately	128	46
	Poorly	52	19
	Undifferentiated	2	1
	Unknown	6	—
	Localisation		
	Tongue/oral cavity	49	17
	Floor of mouth	43	15
	Oropharynx	42	15
	Hypopharynx	94	33
	Larynx	54	19
Treatment			
	Surgery alone	79	28
	Surgery + RT postoperative	173	61
	Surgery + RT + chemotherapy	30	11

RT, radiotherapy.

Tumours were classified histologically according to the UICC classification [12]. The main clinicopathological characteristics of the cases are listed in Table 1. The local invasiveness was scored according to the histologically determined pattern and extent of infiltration to contiguous anatomical structures [13]. Because of the high incidence of hypopharyngeal tumours in the eastern part of France, tumours of the hypopharynx, mainly piriform sinus, were predominant, and all other tumour sites were equally represented.

Determination of 11q13 amplification

Tissue samples were obtained immediately after tumour resection and stored in liquid nitrogen until DNA preparation. They included 282 tumour samples, 144 involved lymph nodes (116 were matched to the tumour) and 250 normal tissue samples from the same patients.

Amplification of *HST-1/FGF-4* and *BCL-1* genes was evaluated by Southern blot analysis and quantified by slot blot analysis as previously described [11]. Briefly, it consisted of *Eco*RI digestion of genomic DNA, electrophoresis and Southern blot, hybridisation with specific ^{32}P-labelled probes, i.e. *HST-1/FGF4* and *BCL-1* on 11q13. The control probes comprised a marker on chromosome 8, *C-MOS*, which serves as an index of relative single copy gene number to ensure uniform DNA loading and two markers on 11q, distal to the common site of amplification at 11q13, progesterone receptor (PgR) and *C-ETS-1* at 11q22-23, to rule out the possibility of multiple copy numbers of chromosome 11.

The levels of *HST-1/FGF-4* and *BCL-1* gene amplification were quantified by densitometric analysis (with a BIOCOM image analyser) of autoradiograms of slot blots of the same DNA samples serially diluted. Care was taken to correct for film non-linearity, unequal DNA loading and efficiency of hybridisation, as previously described [11]. The oncogene copy number of the tumour tissue samples was determined according to the normal tissue sample. Gene amplification was defined as an increase of twice the normal gene dosage. In cases where amplification was low-level (<3), DNA samples were analysed on at least three separate blots for confirmation.

DNA probes

HST-1/FGF-4: 0.78 kb *Eco*RI-*Sst*1 fragment from the CS1 clone [14], *BCL-1*: 2.3 kb *Sst1* fragment from pRc8smR [3], *C-MOS*: 2.5 kb *Eco*RI fragment from pHM2A (HGM 10), *C-ETS-1*: 5.4 kb *Eco*RI fragment from clone pHE 5.4 [15], *PgR*: 2.1 kb *Bam*H1-*Hind*III fragment of pSG5 [16].

Statistical methods

Associations between amplification of the 11q13 DNA markers, *HST-1/FGF-4* and *BCL-1* and the clinicopatho-

logical variables were assessed using the chi-squared test. The statistical analyses were performed using the BMDP and the STATXACT statistical software package for exact non-parametric inference.

Crude survival and event-free survival curves, starting from the date of surgery, were estimated using the Kaplan–Meier method [17]. The equality of the survival distributions was tested by the log-rank test [18]. The inter-relationship between univariate prognostic factors was taken into account and the remaining independent effects on survival estimated by a Cox proportional hazards model, allowing for interaction [19]. Patients who died without evidence of recurrence were censored at the last follow-up in the analysis of event-free survival. For all statistical analyses, a P value of <0.05 was considered statistically significant.

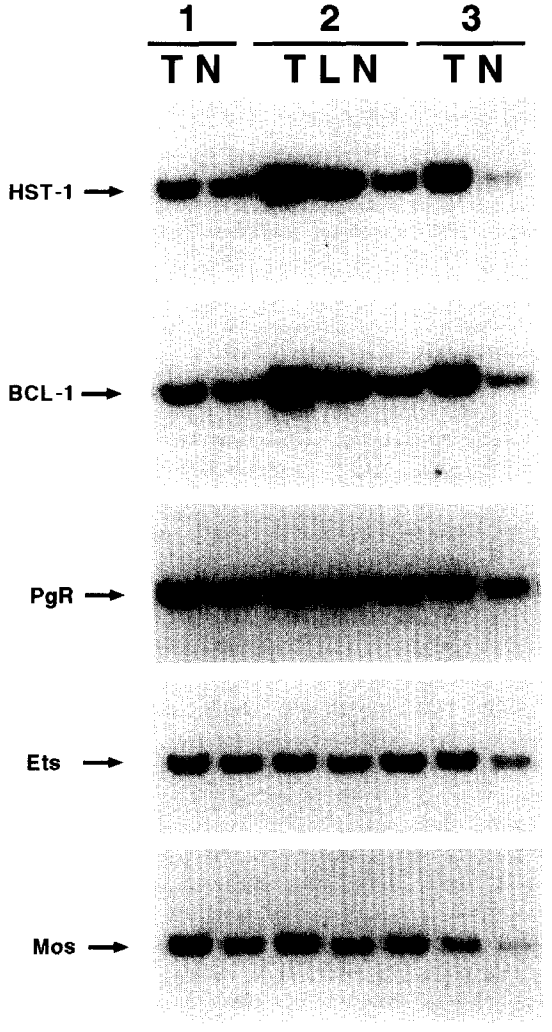

Figure 1. Southern blot analysis of *Eco*RI-digested DNAs from head and neck carcinoma (T), adjacent normal mucosa (N) and metastatic lymph node (L) sample of the same patient. Filters were sequentially hybridised with the *HST-1/FGF-4*, *BCL-1* and *C-MOS* probes, stripped and hybridised with the progesterone receptor (*PgR*) and *C-ETS1* probes. Each vertical lane corresponds to the same DNA filter. The figure shows coamplification (3-fold) of *HST-1/FGF-4* (6.0 kb) and *BCL-1* (9.5 kb) for patient 2 in concomitant primary tumour and lymph node metastasis, for patient 3 in tumour and absence of amplification for patient 1. The size of the bands revealed by *PgR*, *ETS-1* and *C-MOS* probes were, respectively, 6.0 kb, 6.5 kb and 2.5 kb.

RESULTS

11q13 Amplification and tumour characteristics

In this study, amplification of *HST-1/FGF-4* and *BCL-1* genes was evaluated in 282 primary tumours by Southern and slot blots. An example of amplification is shown in Figure 1. Overall, *HST-1/FGF-4* gene amplification was detected in 52% (146/282) of tumours. The frequency was lower in the women (8/25 =32%) compared to men (54%) (P=0.038). The incidence was significantly higher in tumours arising in the hypopharynx (76%) compared to the other sites (40%) (Table 2, P=0.0007). The *BCL-1* locus was coamplified with *HST-1/FGF-4*, except for six cases. Amplification of the *cyclin D1/PRAD1* gene analysed in 66 cases was found coamplified as well, even in those six cases. The degree of *HST-1/BCL-1* amplification ranged from 2- to 18-fold (median value 4.7-fold). Amplification of the 11q13 DNA markers was detected in 55.5% of involved lymph nodes. As previously described [11], the amplification patterns were similar in the matched involved lymph nodes and primary tumours.

Table 2 shows the relation between *HST-1/BCL-1* amplification and clinicopathological parameters. In accordance with our previous results, *HST-1/BCL-1* gene amplification was significantly related to the tumour site (P=0.0007) and also to neck lymph node involvement (P=0.013). The incidence of 11q13 amplification was higher in patients with nodal involvement (57%) in comparison with 37% of node-negative patients. There was a significant association between 11q13 amplification and differentiation status (P=0.02). Moreover, a significant relationship was found between amplification of the *HST-1/FGF-4* gene and clinical stage (P=0.015); however, the relationship was not consistent with increasing clinical

Table 2. Relationship between HST-1/FGF-4 *gene amplification and disease parameters of the head and neck squamous cell carcinomas*

Disease parameters*	Amplified/total (%)	P value†
Tumour localisation		
Base of tongue/tongue	21/49 (43)	
Floor of the mouth	16/43 (37)	
Oropharynx	17/42 (41)	0.0007
Hypopharynx	71/94 (76)	
Larynx	21/54 (39)	
Neck node status		
Negative	24/65 (37)	0.013
Positive	113/198 (57)	
Clinical stage		
I (T_1,N_0,M_0)	9/14 (64)	
II (T_2,N_0,M_0)	8/30 (27)	0.015
III (T_3,N_0,M_0/T_{1-3},N_1,M_0)	21/44 (48)	
IV (T_4,N_{0-1},M_0/T,N_{2-3},M_1)	99/175 (57)	
Differentiation		
Well	41/94 (44)	
Moderate	66/128 (52)	0.02
Poor	37/54 (69)	
Local invasiveness		
Low	24/54 (44)	
Moderate	55/99 (56)	0.33 (NS)
High	53/97 (55)	

Numbers in table are numbers of cases; NS, not significant. *See Patients and Methods for descriptions of the disease parameters. †P value based on the chi-squared test.

stage. No relationship with the tumour size, nor with the local invasiveness pattern of tumours, was found.

11q13 Amplification and relation with clinical outcome

Given that survival of head and neck cancer patients is dependent on treatment modalities, we analysed two distinct groups of patients which are homogeneous with regard to their respective treatments. Thus, out of 282 patients, 203 were treated by surgery followed by radiotherapy while the remaining 79 patients were treated by surgery only.

Two of the 203 patients who had received radiotherapy after surgery were lost to follow-up. The clinical characteristics of this group of 201 patients are noted in Table 3. In this group, 11q13 amplification in tumours was observed in 116 cases (57%).

During the follow-up period, 94 patients had no evidence of progression. Among the 107 patients with progressive disease, 15 (14%) presented local relapse, 28 (26%) lymph node involvement and 31 (29%) distant metastasis, as first progression event. Metastases occurred mostly in the lung ($n = 7$), bone ($n = 9$) or were multiple ($n = 10$). Second primary tumours were observed in 33 cases (31%). As illustrated by the Kaplan–Meier curve (Figure 2a), no significant difference in event-free survival was observed between patients with and without 11q13 amplification in tumour (log-rank test P=0.82). Similarly, as shown in Figure 2b, the 3-year crude survival rates of the 11q13 amplified and non-amplified groups were 48% and 52%, respectively. The log-rank test did not demonstrate significant association between 11q13 amplification and crude survival (P=0.61).

As expected, the multivariate analysis of our series, using Cox's proportional hazard model, showed tumour size and node status to be independent prognostic determinants of the relapse-free and crude survival (Table 4).

Table 3. *Characteristics of the* 201 HNSCC patients included in the survival analysis

	Characteristic	Number of cases	(%)	Number amplified
Patients	Total number	201	(100)	116
Tumours	Size (pathological)			
	pT1	29	(14)	19
	pT2	73	(36)	39
	pT3	73	(36)	42
	pT4	25	(12)	15
	Unknown	1	—	
	Nodal status (pathological)			
	pN0	23	(11)	11
	pN1	27	(13)	18
	pN2a,b,c	129	(64)	70
	pN3	19	(9)	15
	Unknown	3	—	
	Localisation			
	Tongue/oral cavity	32	(16)	15
	Floor of mouth	29	(14)	13
	Oropharynx	35	(17)	16
	Hypopharynx	80	(40)	59
	Larynx	25	(12)	13
Outcome				
	Dead	85	(42)	51
	Progression	107	(53)	63
	Without progression	94	(47)	53

In the selected subgroup of 79 patients treated by surgery without adjuvant therapy, the incidence of 11q13 amplication in tumours was much lower than in the whole series of patients (35% versus 52%), probably due to the lower frequency of hypopharyngeal tumours (20%). The univariate analysis revealed no significant difference in event-free survival (Figure 3a) or crude survival (Figure 3b) between patients with tumours with or without *HST-1/BCL-1* amplification, although survival was slightly shorter for patients with amplified tumours.

Since tumour location is considered to be an independent predictor of survival of head and neck cancer patients, we analysed the survival of patients with hypopharyngeal tumours, which was the largest tumour site group in our series ($n = 80$). Crude survival of this subset of patients was not different to the crude survival of the whole group of patients. Additionally, with regard to 11q13 amplification, the 3-year survival rates of the patients with hypopharyngeal tumours were not significantly different (data not shown).

DISCUSSION

Gene amplification is one of the mechanisms for activating oncogenes by increasing the copy number of the genes potentially involved in tumour progression. The majority of head and neck tumours show coamplification of the *INT-2/FGF-3*, *HST-1/FGF-4* and *BCL-1* genes which are closely linked to the *CCND1* gene, which is presumed to have an oncogenic role since it is found overexpressed in tumour tissues [10,20]. Thus, amplification of the *HST-1* gene and *BCL-1* gene may be regarded as markers indicative of possible amplification of any one of the genes of the 11q13 amplicon. To some extent, our results, which update our previous report on 11q13 gene amplification in human head and neck tumours [11] and investigate its significance in clinical outcome, strengthen the possible implication of genes of the 11q13 region in malignant disease.

In this large study, 11q13 amplification in head and neck tumours occurred in 52% of cases with a range of 37–76% depending on the tumour site. The highest incidence, as well as level of 11q13 amplification, was predominantly observed in the hypopharyngeal tumours, in agreement with two other studies [21,22]. An incidence of 35% has generally been reported in other data [21,23,24] consistent with the incidence of 11q13 amplification we observed in the tumours of sites other than the hypopharynx. Incidences as low as 7% [25] or 12.5% [26] have also been reported. The criteria used to define amplification (hybridisation probes, controls to correct for polysomy and DNA loading, etc.) as well as the different sites of the tumours studied may account for the variability of the reported prevalences. Thus, in HNSCCS, 11q13 amplification is a prominent genetic alteration similar to the tumours of the oesophagus where 11q13 amplification incidence was 30–40% [6,27], while the incidence was 15–25% in breast cancers [4,28], 3% in ovarian cancers [29] and in any colorectal carcinomas [30].

This study confirms, together with the correlation between 11q13 amplification and tumour site, the strong positive relationship between 11q13 amplification and lymph node status, found in our previous study [11]. These results are also in agreement with two published reports with a lower number of patients [23,24] (Table 5). Our data confirmed a

relationship with tumour differentiation [5], but did not confirm the correlation of 11q13 amplification with high local invasiveness [21, 23]. The localisation of the tumours studied in larynx [23] as well as the criteria used to evaluate the pattern of invasiveness may explain the lack of consensus. 11q13 amplification can be considered as the hallmark of potentially aggressive head and neck tumours. This prominent event occurring frequently in head and neck tumours may contribute to the emergence of aggressive tumours, probably by conferring a growth-selective advantage and clonal expression capabilities to cells. This hypothesis is supported by the observation that matched primary tumours and involved lymph nodes present similar 11q13 alterations, suggesting that the 11q13 amplification pattern acquired by a primary tumour is maintained throughout head and neck cancer dissemination [11]. However, amplification of 11q13 DNA markers was found to be of no predictive value for loco-regional recurrence, which is the major progressive feature of head and neck cancer patients. Moreover, due to continuous exposure to the main known risk factors tobacco and alcohol, head and neck cancer patients have the propensity to develop multiple primary cancers either synchronous or metachronous [31], as well as second primary cancers in the same site or in others. In one study [32], 11q13 alterations have been related to tumours with multiple localisations. In our study, this association was also observed, but without reaching statistical significance. Nevertheless, 11q13 amplification was of no prognostic value with regard to the occurrence of second primary cancer.

The clinical outcome of head and neck cancer patients is thought to be dependent on treatment modalities whch may

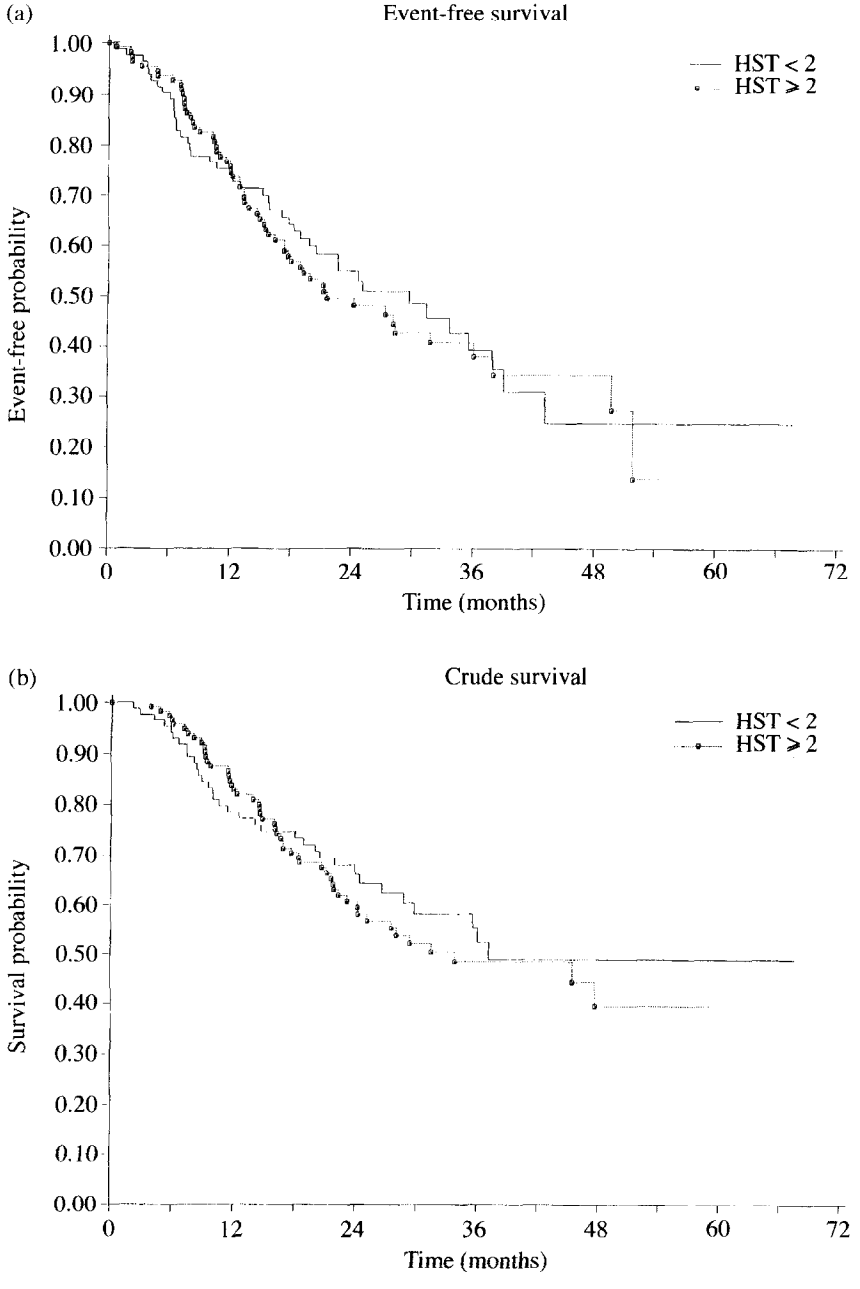

Figure 2. Survival curves (Kaplan–Meier) of 201 head and neck cancer patients, treated by surgery and postoperative radiotherapy, with (HST ≥2) and without (HST<2) amplification of *HST-1 / BCL-1* genes. No significant differences were found for (a) event-free survival rates (log-rank test, P=0.82) and (b) crude survival rates (log-rank test, P=0.61).

Table 4. Multivariate analysis of crude survival in 201 head and neck cancer patients

Parameters	RR (95%CI)*	*P* Value
Tumour size		
pT_3 versus pT_1–T_2	2.2 (1.4–3.6)	0.0001
pT_4 versus pT_1–T_2	3.5 (1.9–6.3)	
Nodal status (positive versus negative)	2.3 (0.9–5.6)	0.04
HST-1 amplification (negative versus positive)	1.0 (0.7–1.6)	0.89

*Hazard rate ratio with 95% confidence interval.

have different effects on improving long-term survival. This analysis of the largest group of patients so far reported (201 cases) treated in a single institution by well-standardised curative protocols, i.e. surgery and postoperative radiotherapy, did not demonstrate any prognostic value of 11q13 amplification. This absence of a relationship between 11q13 amplification and patients' survival was also observed in the surgically treated group, and whatever the site of the tumour considered. These results contrast with two recent conflicting reports [34, 35]. In one of these studies, amplification of the *cyclin D1* gene found in 17% of 51 primary laryngeal cancers was related to shortened overall survival, but not event-free survival [34]. The second study [35] described amplification of 11q13 DNA markers in 39% of 56 HNSCCs and a

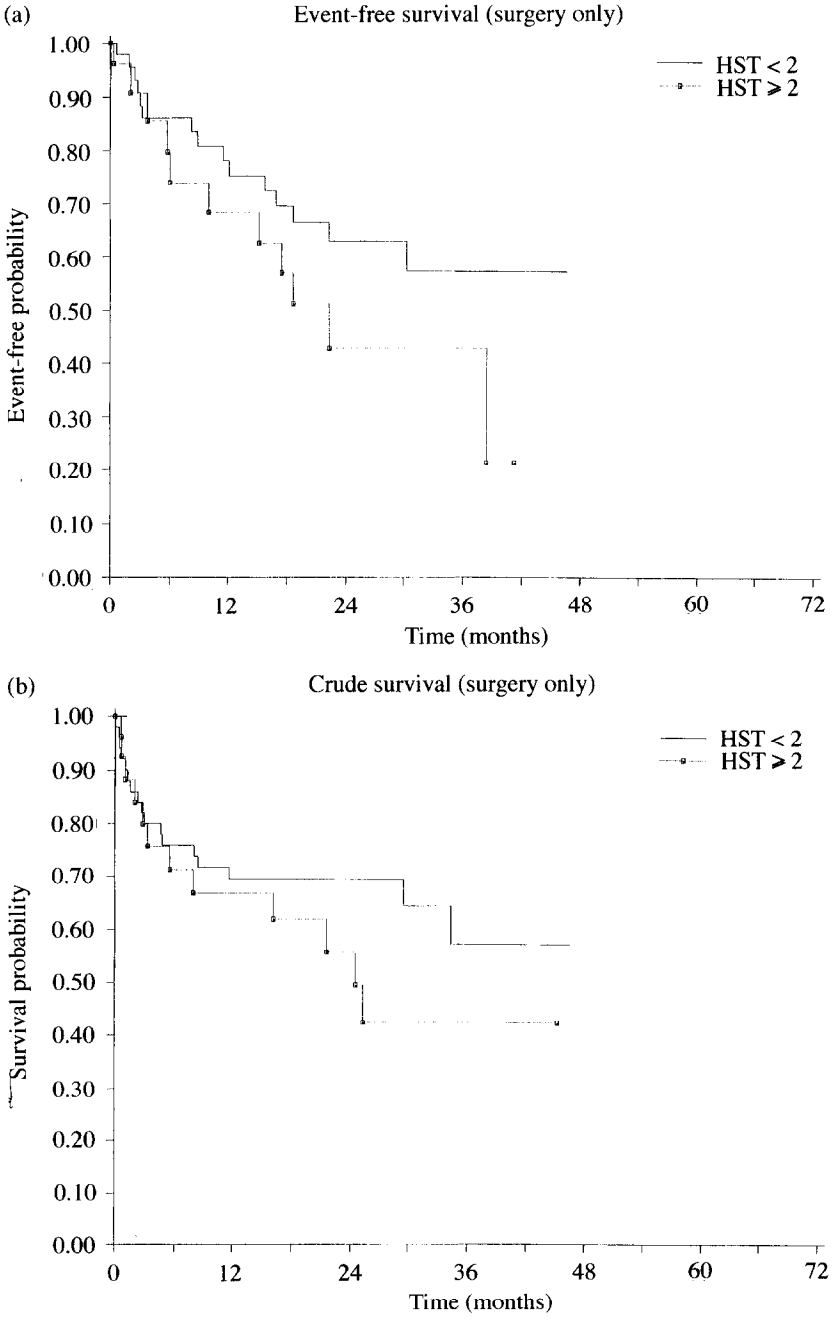

Figure 3. Kaplan–Meier life table analysis for (a) event-free survival; and (b) crude survival of 79 head and neck carcinoma patients without postsurgical treatment with regard to 11q13 amplification.

Table 5. Amplification of 11q13 genes in primary human head and neck cancers

Reference	No. of cases studied	% Amplification	Significant clinical correlation with 11q13 alteration
[22]	47	64* (cyclin D1) (79% hypopharynx)	Tumour site, overall and event-free survival
[23]	46 (larynx)	37 (cyclin D1)	Local invasiveness, lymph node involvement, advanced stage
[24]	32 (larynx)	34 (cyclin D1)	Proliferative index, lymph node involvement
[26]	40	12.5 (hst, bcl 1)	Advanced stage
[32]	51	54 (cyclin D1)	Small size, multiple primary tumours
[34]	51 (larynx)	17.6 (cyclin D1)	Overall survival
[21,35]	56	39 (hst-1, bcl-1, cyclin D1) (68% hypopharynx)	Local invasiveness, tumour site, incidence of recurrence or persistence of disease, overall survival
[37]	64	44* (cyclin D1)	Tumour site, no relation with survival
Present study	282	52 (hst-1, bcl-1) (78% hypopharynx)	Tumour site, lymph node involvement, no relation with survival

*Immunohistochemical study.

significant relationship between this amplification and event-free and overall survival. The clinical course of this rather small cohort did not correlate with the established clinical risk factors such as lymph node status or tumour size. Moreover, it included patients who were given various types of treatment such as surgery or radiotherapy or a combination of both, which cannot exclude introduction of a bias in the estimation of survival. Two other reports in which *cyclin D1* protein overexpression was examined immunohistochemically, also produced conflicting results. In a retrospective study of 47 selected HNSCCS patients, cyclin D1 overexpression was related to the hypopharyngeal tumour site and to reduced event-free survival and overall survival [22] whilst, in contrast, the other reported an absence of correlation [36]. Similar contradictory data have been reported for cancer of the oesophagus [37–39] and breast [28, 40, 41] although no prognostic significance of *cyclin D1* gene has been demonstrated in two large breast cancer studies [41, 42] and in primary non-small cell lung cancer [43].

The mechanism by which *cyclin D1* might contribute to the progression of tumours, if any, still remains unknown. Compelling evidence of interaction between D-type cyclins and the retinoblastoma gene, a negative regulator of cell proliferation [44] or the tumor suppressor gene P53 [45] suggests the involvement of *cyclin D1* in complex pathways, such as apoptosis. Additionally, deregulation of transitions through key checkpoints of the eukaryotic cell cycle might contribute to malignant transformation/proliferation [46]. Recently, the existence of two forms of cyclin D1 mRNA have been demonstrated, suggesting a splicing mechanism between exon 4 and exon 5 modulated by a polymorphism in the splice donor region [47]. Interestingly, preliminary results have reported that one of the variants was correlated with a greater risk of local relapse in lung tumour patients. [47] Investigation of this alternative spliced form may be a relevant target in the study of head and neck cancer progression.

1. Tomatis, L, Aitio A, Day NE, *et al. Cancer: Causes, Occurrence and Control.* Lyon, IARC Scientific Publications, 1990, 100.
2. Arnold A, Kim HG, Gaz RD, *et al.* Molecular cloning and chromosomal mapping of DNA rearranged with the parathyroid hormone gene in a parathyroid adenoma. *J Clin Invest* 1989, **83**, 2034–2040.
3. Tsujimoto Y, Jaffe E, Cossman J, Gorham J, Nowell PC, Croce CM. Clustering of breakpoints on chromosome 11 in human B-cell neoplasms with the t(11–14) chromosome translocation. *Nature* 1985, **315**, 340–343.
4. Lidereau R, Callahan R, Dickson C, Peters G, Escot C, Ali IU. Amplification of the int-2 gene in primary human breast tumors. *Oncogene Res* 1988, **2**, 285–291.
5. Berenson JR, Yang J, Mickel RA. Frequent amplification of the bcl-1 locus in head and neck squamous cell carcinomas. *Oncogene* 1989, **4**, 1111–1116.
6. Kitagawa Y, Ueda M, Ando N, Shinozawa Y, Shimizu N, Abe O. Significance of int-2/hst-1 coamplification as a prognostic factor in patients with esophageal squamous carcinoma. *Cancer Res* 1991, **51**, 1504–1508.
7. Berenson JR, Koga H, Yang J, Pearl J, Holmes E, Figlin R. Frequent amplification of the Bcl-1 locus in poorly differentiated squamous cell carcinoma of the lung. *Oncogene* 1990, **5**, 1343–1348.
8. Motokura T, Arnold A. Prad1/cyclin D1 proto-oncogene: genomic organization, 5′ DNA sequence, and sequence of a tumor-specific rearrangement breakpoint. *Genes Chrom Cancer* 1993, 7, 89–95.
9. Lammie GA, Fantl V, Smith R, *et al.* D11S287 a putative oncogene on chromosome 11q13 is amplified and expressed in squamous cell and mammary carcinomas and linked to bcl-1. *Oncogene* 1991, **6**, 439–444.

10. Motokura T, Arnold A. Cyclin D and oncogenesis. *Curr Opin Gen Dev* 1993, **3**, 5–10.
11. Muller D, Millon R, Lidereau R, *et al.* . Frequent amplification of 11q13 DNA markers is associated with lymph node involvement in human head and neck squamous cell carcinomas. *Eur J Cancer Oral Oncol* 1994, **30b**, 113–120.
12. U.I.C.C. *TNM Classification of Malignant Tumours*, 4th edition. P. Hermaneck and L.H. Sobin, eds. Berlin, Springer 1987.
13. Muller D, Wolf C, Abecassis J, *et al.* Increased stromelysin-3 gene expression is associated with increased local invasiveness in head and neck squamous cell carcinomas. *Cancer Res* 1993, **53**, 165–169.
14. Adelaide J, Mattei MG, Marics I, *et al.* Chromosomal localization of the hst oncogene and its co-amplification with the int-2 oncogene in a human melanoma. *Oncogene* 1988, **2**, 413–416.
15. De Taisne C, Gegonne A, Stehelin D. Chromosomal localization of the human proto-oncogene c-ets. *Nature* 1984, **310**, 581–583.
16. Kastner P, Krust A, Turcotte B, *et al.* Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B. *EMBO J* 1990, **9**, 1603–1614.
17. Kaplan EL, Meier P. Nonparametric estimation from incomplete observations. *J Am Stat Assoc* 1958, **53**, 457–481.
18. Mantel N. Evaluation of survival data and two new rank order statistics arising in its consideration. *Cancer Chemother Rep* 1966, **50**, 163–170.
19. Cox DR, Oakes D. *Analysis of Survival Data*. London, Chapman and Hall 1984.
20. Lovec H, Sewing A, Lucibello FC, Muller R, Moroy T. Oncogenic activity of cyclin D1 revealed through co-operation with Ha-ras: link between cell cycle control and malignant transformation. *Oncogene* 1994, **9**, 323–326.
21. Williams ME, Gaffey MJ, Weiss LM, Wilczynski SP, Schuuring E, Levine PA. Chromosome 11q13 amplification in head and neck squamous cell carcinoma. *Arch Otolaryngol, Head Neck Surg* 1993, **119**, 1238–1243.
22. Michalides R, Vanveelen N, Hart A, Loftus B, Wientjens E, Balm A. Overexpression of cyclin D1 correlates with recurrence in a group of forty-seven operable squamous cell carcinomas of the head and neck. *Cancer Res* 1995, **55**, 975–978.
23. Jares P, Fernandez PL, Campo E, *et al.* . PRAD-1/cyclin D1 gene amplification correlates with messenger RNA overexpression and tumor progression in human laryngeal carcinomas. *Cancer Res* 1994, **54**, 4813–4817.
24. Callender T, Elnaggar AK, Lee MS, Frankenthaler R, Luna HA, Batsakis JG. PRAD-1 (CCND1) cyclin D1 oncogene amplification in primary head and neck squamous cell carcinoma. *Cancer* 1994, **74**, 152–158.
25. Leonard JH, Kearsley JH, Chenevixtrench G, Hayward NK. Analysis of gene amplification in head and neck squamous cell carcinomas. *Int J Cancer* 1991, **48**, 511–515.
26. Volling P, Jungehulsing M, Stutzer H, Diehl V, Tesch H. Analysis of proto-oncogenes in squamous cell carcinomas of the head and neck. *Int J Oncol* 1993, **3**, 671–677.
27. Tsuda T, Tahara E, Kajiyama G, Sakamoto H, Terada M, Sugimura T. High incidence of coamplification of hst-1 and int-2 genes in human esophageal carcinomas. *Cancer Res* 1989, **49**, 5505–5508.
28. Henry JA, Hennessy C, Levett DL, Lennard TWJ, Westley BR, May FEB. Int-2 amplification in breast cancer—association with decreased survival and relationship to amplification of c-erbB-2 and c-myc. *Int J Cancer* 1993, **53**, 774–780.
29. Courjal F, Louason G, Speiser P, Katsaros D, Zeillinger R, Theillet C. Cyclin gene amplification and overexpression in breast and ovarian cancers: evidence for the selection of cyclin D1 in breast and cyclin E in ovarian tumors. *Int J Cancer* 1996, **69**, 247–253.
30. Leach FS, Elledge SJ, Sherr CJ, *et al.* Amplification of cyclin genes in colorectal carcinomas. *Cancer Res* 1993, **53**, 1986–1989.
31. Jones AS, Morar P, Phillips DE, Field JK, Husband D, Helliwell TR. Second primary tumors in patients with head and neck squamous cell carcinoma. *Cancer* 1995, **75**, 1343–1353.
32. Klijanienko J, Bianchi A.B, Cvitkovic E, Janot F, Luboinski B, Conti CJ. Unexpected Prad-1 amplification in multiple simultaneous localisations of squamous cell carcinoma of the head and neck. *Eur J Cancer* 1993, **29A**, 653–654.
33. Batani JP, Bernier J, Brugere J, Jaulerry C, Picco C, Brunin F. Natural history of neck disease in patients with squamous cell carcinoma of oropharynx and pharyngolarynx. *Radiother Oncol* 1985, **3**, 245–255.
34. Bellacosa A, Almadori G, Cavallo S, *et al.* Cyclin D1 gene amplification in human laryngeal squamous cell carcinomas: prognostic significance and clinical implications. *Clin Cancer Res* 1996, **2**, 175–180.
35. Meredith SD, Levine PA, Burns J, *et al.* Chromosome 11q13 amplification in head and neck squamous cell carcinoma: association with poor prognosis. *Arc Otolaryngol Head Neck Surg* 1995, **121**, 790–994.
36. GaVey MJ, Lezzoni JC, Meredith SD, *et al.* Cyclin D1 (Prad1, CCND1) and glutathione-S-transferase-pi gene expression in head and neck squamous cell carcinoma. *Hum Pathol* 1995, **26**, 1221–1226.
37. Shiga C, Shiga K, Hirayama K, Katayama M, Nishihira T, Mori S. Prognostic significance of hst-1 gene amplification in primary esophageal carcinomas and its relationship to other prognostic factors. *Anticancer Res* 1994, **14**, 651–656.
38. Adelaide J, Monges G, Derderian C, Seitz JF, Birnbaum D. Oesophageal cancer and amplification of the human cyclin D gene CCND1/PRAD1. *Br J Cancer* 1995, **71**, 64–68.
39. Shinozaki H, Ozawa S, Ando N, *et al.* Cyclin D1 Amplification as a new preditive classification for squamous cell carcinoma of the esophagus, adding gene information. *Clin Cancer Res* 1996, **2**, 1177–1184.
40. Schuuring E, Verhoeven E, Vantinteren H, *et al.* Amplification of genes within the chromosome-11q13 region is indicative of poor prognosis in patients with operable breast cancer. *Cancer Res* 1992, **52**, 5229–5234.
41. Seshadri R, Lee CSL, Hui R, McCaul K, Horsfall DJ, Sutherland RI. Cyclin D1 amplification is not associated with reduced overall survival in primary breast cancer but may predict early relapse in patients with features of good prognosis. *Clin Cancer Res* 1996, **2**, 1177–1184.
42. Berns PM, Klijn JG, Staveren IL, Smid M, look MP, Foekens JA. Distinct gene alterations as related with prognosis in human primary breast cancer. *Breast Cancer Res Treat* 1994, **32**, 92.
43. Betticher DC, Heighway J, Hasleton P, *et al.* Prognostic significance of CCND1 (cyclin D1) overexpression in primary resected non-small-cell lung cancer. *Br J Cancer* 1996, **73**, 294–300.
44. Claudio PP, de Luca A, Howard CM, *et al.* Functional analysis of pRb2/p130 interaction with cyclins. *Cancer Res* 1996, **56**, 2003–2008.
45. Chen XB, Bargonetti J, Prives C. P53, through p21 (WAF1/CIP1), induces cyclin D1 synthesis. *Cancer Res* 1995, **55**, 4257–4263.
46. Uchimaru K, Ends K, Fujinuma H, Zukerberg L, Arnold A, Motokura T. Oncogenic collaboration of the cyclin D1 (PRAD1, bcl-1) gene with a mutated p53. 459–465.
47. Betticher DC, Thatcher N, Altermat HJ, Hoban P, Ryder WDJ, Heighway J. Alternate splicing produces a novel cyclin D1 transcript. *Oncogene* 1995, **11**, 1005–1011.

Acknowledgements—We are grateful to Dr. R. Lidereau (Saint Cloud, France) for helpful advice and C. Engels and M. Krieger for technical assistance. We wish to thank Dr. A. Arnold (Boston, Massachusetts, U.S.A.) for providing the Prad1/CCND1 probe, Dr. D. Birnbaum (Inserm U119, Marseille, France) for providing the hst-1 probe, Dr. C. Theillet (URA CNRS 1191, Montpellier, France) for providing the bcl-1 probe. This work was supported by grants from the Fédération Nationale des Centres de Lutte Contre le Cancer and the Comité de la Ligue Régionale du Haut-Rhin.