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Frequent Amplification of 11q13 DNA Markers is Associated with Lymph Node Involvement in Human Head and Neck Squamous Cell Carcinomas

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Amplification of 11q13 DNA markers, particularly *hst*-1/FGF4 oncogene and the *bcl*-1 locus, was evaluated in 178 head and neck squamous cell carcinomas (SCCs) by Southern blot and slot blot hybridisation. Coamplification of *hst*-1/FGF4 and *bcl*-1 genes was found in 57% of primary tumours and in 60% of the 89 metastatic lymph nodes tested. The pattern of amplification was significantly similar in matched sets of primary SCCs and metastatic lymph nodes. Levels of amplification, quantified by densitometric analysis of slot blots, ranged from 2 to 18-fold normal gene dosage. Also, c-*myc* oncogene (8q24) was found amplified less frequently, since 7% of 169 SCCs tested contained amplification of this gene, the level of which ranged from 2 to 8-fold. *Hst*-1/*bcl*-1 gene amplification was observed more frequently in the tumours arising from the hypopharynx. Coamplification of *hst*-1 and *bcl*-1 genes was significantly positively associated with tumours with nodal involvement ($P = 0.001$). Incidence of *hst*-1/*bcl*-1 gene amplification is higher in the tumours with a clinical stage III or IV. *Hst*-1/*bcl*-1 gene amplification was not related to tumour differentiation or local invasiveness. This prospective study shows that amplification of 11q13 DNA markers is a prominent event occurring in head and neck SCC and may contribute to the pathogenesis and evolution of a subset of patients bearing this type of cancer.
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INTRODUCTION

CANCER OF the head and neck, mainly squamous cell carcinoma (SCC) of the larynx, pharynx and oral cavity, has a high incidence in Western countries (47/100 000 inhabitants per year) and a high mortality rate (9/100 000) [1]. Well known risk factors are alcohol consumption and smoking habits. Nevertheless, the incidence of head and neck SCCs is relatively rare with respect to the large number of individuals exposed to these carcinogens, suggesting that host factors of a genetic nature must affect individual disease susceptibility. Tumours of the head and neck manifest a wide range of clinical behaviour among patients with a tumour of the same grade and, treated by an homogeneous combination of surgical and radiation therapy, the response to therapy and the clinical course are variable. Patients treated for SCC of the head and neck suffer principally loco-regional recurrence of the tumour (40–50%) while distant metastases occur at a lower incidence (15–30% of cases). They may also develop second cancer in 10–20% of cases, half of them occurring in the head and neck area.

Genetic alterations, namely activation of cellular proto-oncogenes or inactivation of suppressor genes, are most probably involved in the multistep process of carcinogenesis and the progression of cancer. Knowledge of the molecular biology of head and neck cancer could provide evidence for understanding their biological diversity and allow to identify additional accurate prognostic factors.

Investigation of head and neck SCCs has identified some oncogene activation or suppressor gene inactivation, recently reviewed by Field [2]. So far, mutation or overexpression of the *ras* gene family was described at variable frequency with regard to the geographical origin of head and neck tumour patients [3, 4]. Overexpression of the c-*erb*B1 (EGF receptor) gene [5] and of the c-*myc* oncogene [3], which encodes a 62 000 dalton nuclear protein acting as a transcription factor was detected in 53% and 30% of this type of cancer, respectively. Recently, abnormalities of the p53 suppressor gene have been reported in laryngeal SCCs as the most frequent genetic

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alteration identified in this cancer (60%) [6]. Overexpression of the p53 gene has been correlated with a history of heavy smoking and drinking [7].

Cytogenetic analysis of head and neck SCCs has revealed multiple chromosomal abnormalities including chromosomes 1, 3, 10 and 11 [8]. Particularly, clustering of breakpoints at 11q13 were frequently found [9]. This site includes the closely located *int*-2 and *hst*-1 genes as well as the *bcl*-1 locus which are all within a 250 kb region. Amplification of these genes (either singly or coamplified) has been reported in 33% of lung tumours [10], 15% of breast tumours [11], 40% of oesophageal tumours [12] and in 34% of head and neck tumours [13].

The biological significance of this genetic alteration remains unclear. The *int*-2 gene was described as a proto-oncogene implicated in virally-induced mammary tumours in mice whereas the *hst*-1 gene was first described from NIH 3T3 cells transfected with DNA of a human stomach cancer. Products of both genes belong to the family of the fibroblast growth factors (FGF) which display mitogenic and angiogenic activities [14]. However, expression of *int*-2/FGF3 or *hst*-1/FGF4 genes was generally absent or low in tumours with amplification of these genes [15]. The *bcl*-1 locus defines a translocation breakpoint on chromosome 11 in B-cell proliferative malignancies [16]. Actually, two potentially interesting genes, located within the 11q13 amplification unit, the *Prad*1 gene and the *ems*1 gene were found overexpressed in breast tumours and head and neck SCC cell lines [17].

Consistent with oncogene functions, the significance of oncogene alterations as prognostic indicators has been considered in various cancers and demonstrated for some of them, like N-*myc* amplification in neuroblastoma.

Interestingly, in retrospective studies of oesophageal carcinomas, *hst*-1/FGF4 and *int*-2/FGF3 gene amplification was significantly related to poor clinical outcome, independently of the clinicopathological parameters [18]. Amplification of the 11q13 region was also associated with the occurrence of local recurrence and distant metastases in breast cancer [11, 19]. In head and neck SCC, overexpression of the c-*myc* gene was reported of significant value in predicting patient outcome [20].

This paper aims to document the incidence of the amplification of the *hst*-1/FGF4, *bcl*-1 and c-*myc* oncogenes in head and neck SCCs and the involvement of the alteration of these genes in the different growth and metastatic behaviour of the tumours. Amplification analysis was performed on 178 fresh tumour samples and 89 metastatic lymph node samples from the same patients by Southern blot and slot blot hybridisation analysis. The data was correlated with clinical and pathological parameters.

MATERIALS AND METHODS

Patients and tissue samples

Fresh tissue samples were obtained from 178 patients (163 males and 15 females) undergoing surgery for head and neck tumours, as previously described [21]. In brief, in each case, the portion of the tumour used for gene amplification analysis was resected near the advancing edge of the tumour, avoiding its necrotic centre. Assessment of tumour cellularity was made from an adjacent section and almost all cases were comprised of 60–80% cancer cells. The tumour samples were immediately frozen and stored in liquid nitrogen until DNA extraction. The remaining resected tumour was fixed in formaldehyde and embedded in paraffin for further histological evaluation. The 178 head and neck SCCs comprised: 16 base of tongue, 7 tongue, 10 floor of the mouth, 13 palate tumours and 25 tumours arising from the oropharynx, 74 from the hypopharynx and 33 from the larynx. None of the patients had previously received radio or chemotherapy. In 89 cases, tissue samples of disrupted lymph nodes, corresponding to metastatic masses, were also available for analysis. Normal mucosa of the upper aerodigestive tract, resected 5 cm distant from the tumour area, was obtained in 151 cases.

The pathological and clinical stages of the head and neck SCC were based on the guidelines established by the UICC criteria [22]. The tumour size and nodal status and also the differentiation status were determined histologically. Together, T and N classification determine the overall clinical stage (I–IV). Moreover, we take into account the degree of local infiltration as described previously [21]. Briefly, the invasive features of the tumours were scored on an arbitrary scale, as low (+), moderate (++) and high (+++), according to the histologically determined extent of infiltration to contiguous anatomic structures.

Analysis of gene amplification by Southern and slot blot

Amplification of *hst*-1, *bcl*-1 and c-*myc* genes was evaluated by Southern blot analysis and quantified by slot blot analysis for 178 primary head and neck squamous cell carcinomas, 89 metastatic lymph nodes and 151 normal tissues.

High molecular weight DNA was isolated from tissue samples according to standard procedure by proteinase K digestion, phenol extraction and ethanol precipitation [23]. The DNA samples (10 μg) were digested with *Eco*RI, fractionned on 0.8% agarose gels and transferred onto nylon membranes (Hybond N^+, Amersham), after depurination and denaturation as recommended by the manufacturer. The nylon membranes were prehybridised and hybridised at 42°C in 50% formamide, 5 × SSC (1 × SSC is 0.15 mol/l NaCl, 15 mmol/l sodium citrate), 0.5% sodium dodecyl sulphate (SDS), 5 × Denhardt's solution (0.1% polyvinylpyrrolidone, 0.1% Ficoll, 0.1% bovine serum albumin) and 150 μg/ml salmon sperm DNA. After a 16-h hybridisation, the membranes were washed twice at room temperature in 2 × SSC, 0.1% SDS, twice at 65°C in 0.2 × SSC, 0.1% SDS and then in 0.1 × SSC, 0.1% SDS and finally exposed to Kodak XAR films at −80°C with intensifying screens. For rescreening membranes, the hybridised probe was removed by incubating the membranes for 30 min in NaOH 0.4 mol/l at 42°C. Removal of the probe was determined by autoradiography. The cDNA probes were labelled with [alpha-^{32}P]-dCTP by random primer extension (Boehringer Mannheim) to a specific activity of 2×10^9 dpm/μg and added at 10^6 cpm/ml hybridisation mix.

Southern blots were hybridised successively for to up to five probes (described below): *hst*-1, *bcl*-1, c-*myc* and three control probes. 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, which make it possible to rule out the possibility of multiple copy numbers of chromosome 11.

Intensity of the hybridisation signals was determined densitometrically with an image analyser.

For slot blot analysis, DNA was applied at 2-fold dilution beginning with 5 μg DNA, on nylon membranes (Hybond N^+, Amersham) with a vacuum slot blot apparatus (Millipore). The membranes were then treated as described above for Southern blots.

The levels of *hst*-1, *bcl*-1 or c-*myc* gene amplification were quantified by densitometric analysis of autoradiograms of the slot blots. To correct for film non-linearity, the amount of radioactivity bound in each slot was estimated from the optical density of the autoradiographic band by reference to a calibration curve obtained from the densitometric data of a serial dilution of a ^{32}P-labelled standard sample. All results were corrected for DNA loading by densitometric data obtained for the c-*mos* signals. Variation in specific activity of the probe and in efficiency of hybridisation was determined by the inclusion in each slot, of a DNA-standard sample (BT474 cell line DNA, 1.6-fold amplified for *hst*-1/FGF4). The oncogene copy number of the tumour tissue samples was determined according to the normal tissue sample. Gene amplification was defined as an increase 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/FGF4: 0.78 kb *Eco*RI-Sst1 fragment from the CS1 clone [24], *bcl*-1: 2.3 kb Sst1 fragment from pRc8smR [16], c-*mos*: 2.5 kb *Eco*RI fragment from pHM2A (HGM 10), c-*myc*: 1 kb Pst1 fragment containing a portion of exon 2 and exon 3 from pRyc 7.4 [25], c-*ets*-1: 5.4 kb *Eco*RI fragment from clone pHE 5.4 [26], PGR: 2.1 kb *Bam*H1-*Hind*III fragment of pSG5 [27], *Prad*1: 1.7 kb *Eco*RI-*Bam*H1 fragment of pDY-5 (probe B) [28].

Statistical methods

Associations between 11q13 DNA markers amplification and the clinico-pathological variables were assessed using the χ^2 test. This test was performed to compare the presence or absence of *hst*-1/FGF4 gene amplification and the different parameters. The Kruskal–Wallis test [29] was used to determine the relationship between the level of *hst*-1/FGF4 and *bcl*-1 gene amplification and the various parameters cited above. P values of <0.05 were considered statistically significant. The statistical analyses were performed using the STATXACT statistical software package for exact non-parametric inference.

RESULTS

Incidence of hst-1/*FGF*4, bcl-1 *and* prad1 *gene amplification*

A total of 178 DNA samples from primary head and neck SCCs and 89 DNA samples from concomitant involved lymph nodes were studied for *hst*-1/FGF4 and *bcl*-1 gene amplification with comparison to the DNA from the corresponding normal tissue samples (151 cases). Representative results of the Southern blot analysis of *Eco*R1-digested DNA are displayed in Fig. 1. *Hst*-1/FGF4 and *bcl*-1 genes were detected as a 6 kbp and 9.5 kbp hybridising band, respectively. The amplification of the *hst*-1/FGF4 and *bcl*-1 genes was checked by normalising the *hst*-1/FGF4 or *bcl*-1 hybridisation signal to the control probe c-*mos*. C-*ets*-1 and PGR probes were used to show that the increase in *hst*-1/FGF4 or *bcl*-1 copy number was the result of specific gene amplification and not simply chromosomal reduplication. No coamplification of c-*ets*-1 and Pgr genes was observed. Each gene was found amplified in 3 separate cases only. 2 patients carried the previously described 5 kbp-c-*mos* allelic band [30, 31] in both their tumour and normal DNA (data not shown).

Hst-1/FGF4 gene amplification was detected in 58% (103/178) of tumours (Table 1). The degree of *hst*-1/FGF4 gene amplification, quantified by densitometric analysis of slot blots of serially diluted DNA samples of all tumours, compared to the values obtained for DNA of normal tissue and normalised to the c-*mos* hybridisation values (Fig. 2) ranged from 2 to 18-fold (median value is 3.5-fold). The *bcl*-1 gene was amplified at equivalent degrees (Fig. 1) in 101 out of 178 (57%) primary tumour samples tested (Table 1). Coamplification of *hst*-1/FGF4 and *bcl*-1 genes concerned almost all *hst*-1/FGF4 amplified tumours since amplification of *hst*-1/FGF4 gene (2.8 and 6.8-fold) without amplification of *bcl*-1 gene (Fig. 1b) was found in 2 cases. An altered restriction pattern was detected for *bcl*-1 gene in four tumour samples, which were also amplified. An additional hybridising band was observed in Southern blot analysis of *Eco*R1 and *Hind*III digested DNA from these tumours, and the additional band was absent in the corresponding normal DNA, indicating that the rearrangement is tumour specific (data not shown). No somatic rearrangements of the *hst*-1/FGF4 gene were observed in our series. No amplification of the *hst*-1/FGF4 and *bcl*-1 genes was detected in the 151 normal tissue samples tested (Fig. 1).

*Prad*1 gene amplification was assessed in a subset of 44 tumours. Amplification of *Prad*1 gene was detected in all tumours which displayed *hst*-1/FGF4 gene amplification (33 cases), including the two tumours without coamplification of *bcl*-1 (Fig. 1b). Absence of *Prad*1 gene amplification was observed in the 11 tumours out of the 44 studied which do not contain *hst*-1/FGF4 gene amplification (Fig. 1b).

Out of 89 metastatic lymph nodes tested, 59% showed amplification of *hst*-1/FGF4 gene. As shown in Table 2 and Fig. 1, the pattern of *hst*-1/FGF4 and *bcl*-1 gene amplification was similar to the corresponding primary tumour one. Lymph nodes without amplification of *hst*-1/FGF4 gene always corresponded to tumours without amplification (31 cases). Concomitant amplification of *hst*-1/FGF4 gene in the primary tumour and in the involved lymph node was noted for 53 out of 58 cases. Moreover, lack of coamplification of *hst*-1/FGF4 and *bcl*-1 genes was retrieved in the metastatic lymph node of the patients whose tumour bears this pattern (2 cases) (Fig. 1b). In the same way, rearrangement of *bcl*-1 gene was also found in the metastatic lymph node of the 2 patients whose primary tumour displayed it. The pattern of the *Prad*1 gene amplification follows that of the *hst*-1/FGF4 one (12 amplified and four non-amplified cases) (Fig. 1b).

Incidence of c-myc *gene amplification*

Amplification of c-*myc* gene was studied in 169 fresh head and neck SCC samples, by Southern and slot blots. The c-*myc* locus was detected as a 12 kbp band and specific amplification was confirmed using the non-amplified c-*mos* probe also located on chromosome 8. Twelve tumours (7%) showed amplification of this oncogene (Table 1), the level of which ranged from 2 to 8-fold (Fig. 2). Nine of the 12 c-*myc*-amplified tumours were also amplified for *hst*-1/FGF4 and *bcl*-1 genes (Fig. 2).

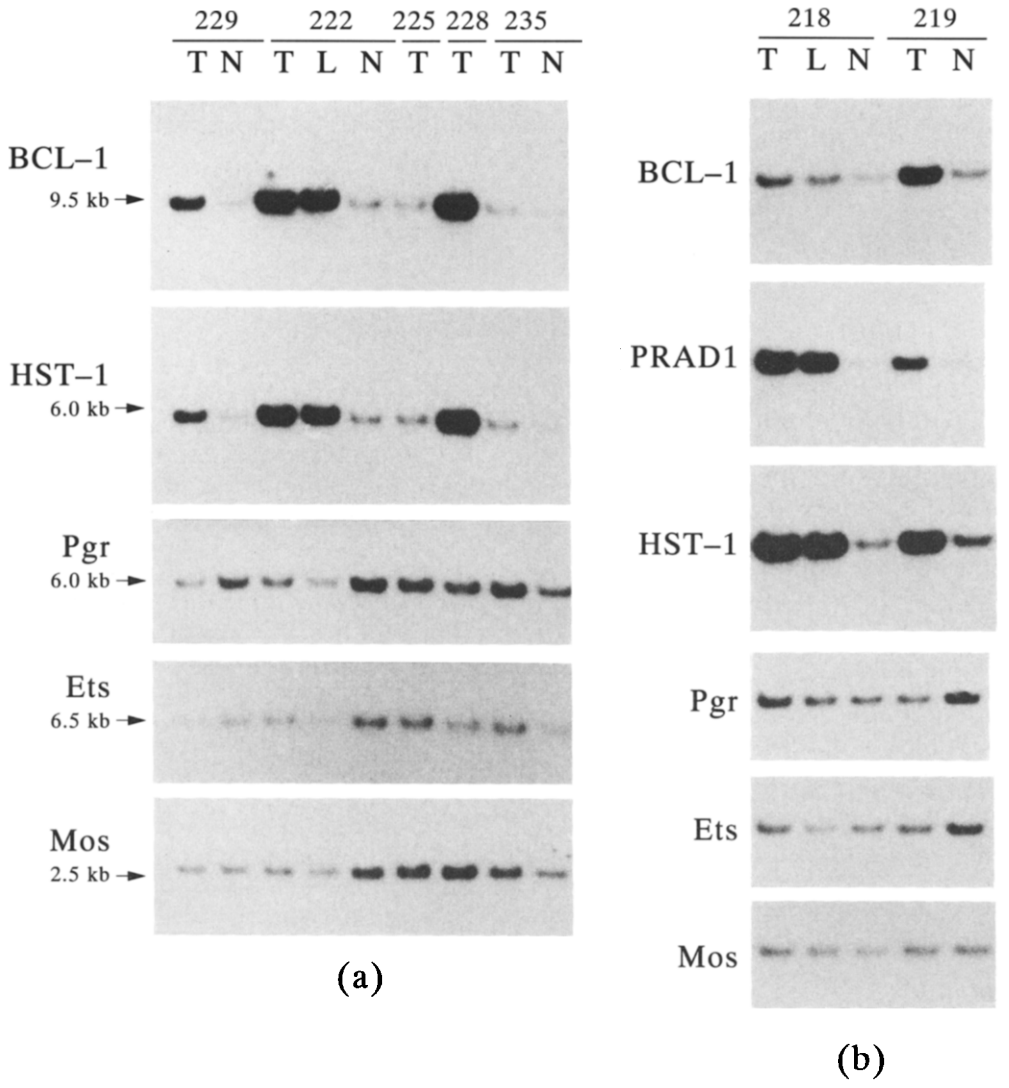

Fig. 1. Southern blot analysis of *Eco*RI-digested DNAs from head and neck carcinoma, adjacent normal mucosa and metastatic lymph node sample of the same patient. Filters were sequentially hybridised with the *hst*-1/FGF4, *bcl*-1 and c-*mos* probes, stripped and hybridised with the progesterone receptor (Pgr) and c-*ets*1 probes. Results are represented in the presumed order of the loci on chromosome 11. Each vertical lane corresponds to the same DNA filter. (a) The figure shows coamplification of *hst*-1/FGF4 and *bcl*-1 for patients 229, 222 and 228, absence of amplification for patients 225 and 235. (b) Coamplification of *hst*-1/FGF4 and *Prad* 1 (one band at 2.5 kb) genes in absence of amplification of *bcl*-1 in the primary tumour and the metastatic lymph node of patient 218. Tumour of patient 219 shows coamplification of *hst*-1/FGF4, *bcl*-1 and *Prad* 1 genes. Numbers are case number. T, tumour DNA, L, metastatic lymph node DNA, N, normal tissue DNA.

C-*myc* gene amplification in metastatic lymph nodes was noted in 6 out of 84 cases tested. For 5 of the 6 cases, the primary tumour was also amplified for c-*myc* gene (Fig. 2).

Table 1. *Amplification of* 11q13 *DNA markers and* c-myc *oncogene in head and neck squamous cell carcinomas*

Loci	Non-amplified*	Amplified†	Total
hst-1/FGF4 (11q13)	75 (42)	103 (58)	178 (100)
bcl-1 (11q13)	77 (43)	101 (57)	178 (100)
c-*myc* (8q24)	157 (93)	12 (7)	169 (100)

( ) percentages, numbers in the table are numbers of cases.
*Non-amplified tumours display levels lower than 2-fold normal gene dosage.
†Amplified tumours display levels ranging from 2 to 18-fold normal gene dosage.

Relationship with the histoclinical parameters

The clinicopathological parameters were related to the *hst*-1/FGF4 gene amplification, since *hst*-1/FGF4 remains the most consistently amplified marker (Table 3).

A significant relationship was found between *hst*-1/FGF4 gene amplification and the tumour localisation ($P=0.0001$, χ^2 test). Incidence of *hst*-1/FGF4 gene amplification was markedly higher in tumours arising from the hypopharynx (72%) compared with the other localisations (32–48%) (Table 3). Moreover, the levels of *hst*-1/FGF4 gene amplification were significantly different between groups of tumours originating from a different localisation ($P=0.0002$, Kruskal–Wallis test). The hypopharyngeal tumours contained also the highest levels of *hst*-1/FGF4 gene amplification (Fig. 3).

A significant relationship was found between *hst*-1/FGF4 gene amplification and the lymph node status ($P=0.001$, χ^2 test, Table 3). The presence of *hst*-1/FGF4 gene amplification

is positively related to the involvement of lymph nodes, since 65% of node positive tumours contained DNA amplified for *hst*-1/FGF4 in comparison with 35% of node negative tumours. Moreover, amplification of the *hst*-1/FGF4 gene is associated to tumours with advanced clinical stages III and IV ($P=0.019$, χ^2 test, Table 3).

Data indicates also absence of relation between amplification of the *hst*-1/FGF4 gene and the differentiation status, or the local invasiveness pattern of tumours (Table 3).

DISCUSSION

Our investigation of 178 head and neck SCCs by Southern and slot blot analysis for amplification of 11q13 DNA markers demonstrates a 58% incidence of *hst*-1/FGF4 gene amplification. The *bcl*-1 locus is generally coamplified (96% of the *hst*-1 amplified tumours) strengthening the existence of a common amplification unit on the chromosome 11.

Previous investigations, performed until yet on limited number cases, described variable frequencies of 11q13 DNA markers amplification in head and neck SCCs. Amplification of *bcl*-1 gene was first described in eight of 23 head and neck SCCs [13]. Amplification of the *int*-2/FGF3 gene, closely located to the *hst*-1/FGF4 gene, was detected in 40% of 21 primary head and neck SCCs [32] and in 62% of cell lines derived from 34 head and neck SCC samples [33]. In contrast, a 7% incidence of *bcl*-1/*int*-2(FGF3) coamplification has been recently described in a study of 66 Australian patients with head and neck SCCs [34]. These variable incidences could be related, at least in part, to differences in population sampling, for which geographical and/or aetiological parameters may vary. Also, our study comprises the largest number of tumours analysed to date, as well as a wide representative sampling of the different head and neck cancer localisations. Thus, our data indicates a differential incidence of 11q13 amplification according to the localisation of the primary tumour. Tumours arising from the hypopharynx are more likely amplified for 11q13 than the tumours arising from other localisations and represent 40% of the total cases tested. Consequently this could account for increasing the frequency of amplified tumours in the whole group of tumours studied.

c-*myc* gene amplification is detected in 7% of our cases, as previously described [34]. Thus, occurrence of c-*myc* oncogene activation by amplification is relatively infrequent in head and neck SCCs. Results of previous studies [20] and our preliminary study [35] have shown that c-*myc* gene overexpression occurs in 30% of head and neck SCCs suggesting therefore, the involvement of different mechanisms for oncogene activation in this type of cancer.

The most commonly used criteria to determine the prognosis and the therapeutic approach to head and neck cancer are tumour size, localisation and the nodal status.

Analysis of relationships between 11q13 amplification and the histo-clinical parameters of the head and neck SCCs

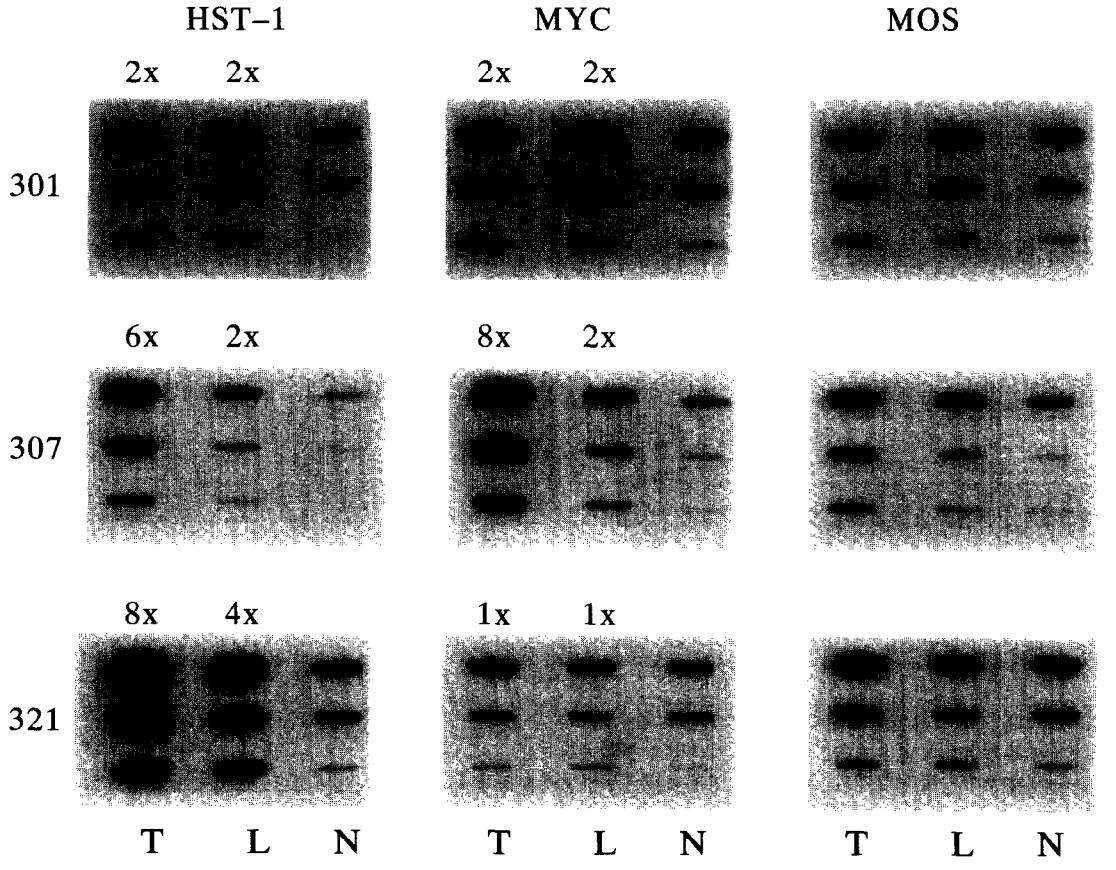

Fig. 2. Example of slot blot analysis of DNA from normal and neoplastic tissue of the head and neck. Filters with three dilutions of each sample (5, 2.5, 1.25 μg DNA) were hybridised with *hst*-1/FGF4 probe, stripped and hybridised with c-*myc* probe, stripped and rehybridised with c-*mos* probe to provide an accurate measure of the amount of DNA loaded in each slot. Numbers above figure are levels of gene amplification (−x) determined by densitometric analysis as described in "Materials and Methods". Numbers are case number. T, tumour DNA, L, metastatic lymph node DNA, N, normal tissue DNA.

Table 2. Comparison between head and neck primary tumour and lymph node metastasis of the same patient with respect to hst-1/*FGF4 gene amplification*

	Tumours	
Lymph node metastasis	Non-amplified*	Amplified†
Non-amplified*	31	5
Amplified†	0	53
Total	31	58

*Non-amplified tumours display levels lower than 2-fold normal gene dosage.
†Amplified tumours display levels ranging from 2 to 18-fold normal gene dosage.
Numbers in the Table are numbers of cases.

Table 3. Relationship between hst-1/*FGF4 gene amplification and disease parameters of the head and neck squamous cell carcinomas*

Disease parameters*	Amplified/Total (%)	*P* value†
Tumour localisation		
Base of tongue	11/23 (48)	
Floor of the mouth	10/23 (43)	
Oropharynx	8/25 (32)	0.0001
Hypopharynx	58/74 (78)	
Larynx	16/33 (48)	
Node status		
Negative	13/37 (35)	0.001
Positive	87/135 (65)	
Clinical stage		
I (T_1, N_0, M_0)	1/4 (25)	
II (T_2, N_0, M_0)	3/15 (20)	
III (T_3, N_0, M_0/T_{1-3}, N_1, M_0)	7/18 (39)	0.019‡
IV (T_4, N_{0-1}, M_0/T, N_{2-3}, M)	48/92 (52)	
Differentiation		
Well	33/63 (52)	
Moderate	44/78 (56)	0.29 (N.S.)
Poor	23/33 (69)	
Local invasiveness		
Low	17/37 (46)	
Moderate	43/73 (59)	0.22 (N.S.)
High	43/68 (63)	

Numbers in the Table are numbers of cases, N.S. = not significant.
*See "Materials and Methods" for descriptions of the disease parameters.
†*P* value based on the χ^2 test.
‡Statistical test applied for comparison of stage I–II and III–IV.

studied demonstrates that amplification of *hst*-1(FGF4)/*bcl*-1 genes occurs more frequently in the hypopharyngeal site of head and neck tumours. In contrast, tumours of the oropharyngeal site are less affected by this alteration. Histological assessment of hypopharyngeal and oropharyngeal tumour tissue samples did not show any significant differences in tumoral cellularity between these two localisations indicating that heterogeneity of the tumoral tissue could not account for this relative difference. Hypopharyngeal tumours are known to be more frequently associated with distant metastases and appear to have the worst prognosis compared to tumours arising from the oral cavity or oropharynx which are rather associated with local recurrence and have a more favourable outcome [36]. Thus, the differential pattern of *hst*-1/FGF4 gene amplification could reflect the differential clinical behaviour of these tumours and points to specific biological properties and/or different molecular mechanisms involved in the growth and evolution of particular head and neck carcinomas.

Furthermore, amplification of 11q13 DNA markers is prevalent in tumours of advanced stage and, particularly, is significantly related to tumours with lymph node involvement. In this way, 11q13 amplification is linked to a parameter which is characteristic of tumour aggressiveness and highly predictive of response to treatment and patient survival. In effect, in head and neck cancer, presence of lymph node metastasis (stage III and IV) is a determinant of patient outcome since rate of recurrent regional disease or of distant metastasis increases with stage of disease.

Hst-1(FGF4)/*bcl*-1 gene amplification is not related to the invasiveness pattern of tumours nor to the differentiation status.

The observation of similar *hst*-1(FGF4)/*bcl*-1 coamplification events in matched primary tumours and metastatic lymph nodes suggests that this genetic alteration takes place prior to the colonisation of distant tissues and thus may occur relatively early in the genesis of the neoplasm. This observation contrasts with that of Adnane *et al.* [37] who reported a low

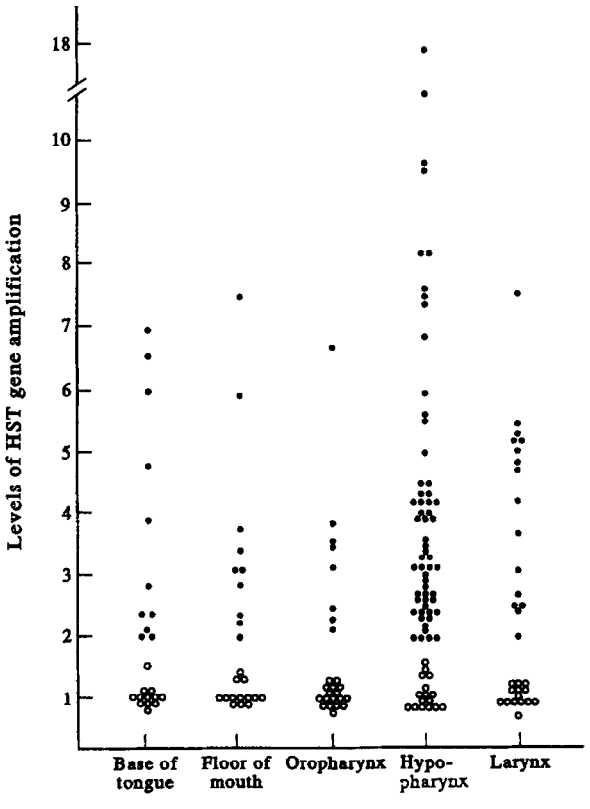

Fig. 3. Levels of *hst*-1/FGF4 gene amplification in head and neck tumours classified according to the tumour localisation. A significant relationship between levels of *hst*-1/FGF4 gene amplification and tumour localisation (*P*=0.0002, Kruskal-Wallis test) was demonstrated. ○ non-amplified tumours, ● amplified tumours.

frequency of concomitant *hst*-1/FGF4 gene amplification in the primary breast tumour and invaded nodes of the same patient (3/13 cases).

Thus, our overall data indicates that 11q13 alteration is a prominent event in head and neck SCC and may play an important role in tumourigenesis and/or tumour progression in head and neck neoplasia by providing tumour cells with a selective growth advantage during tumourigenesis and/or acting as a contributing factor for metastatic spread. The association between amplification of 11q13 DNA markers and lymph node involvement, which has not yet been described in head and neck carcinomas suggest that this alteration could be of clinical and biological significance for head and neck SCC.

The role of the genes (*hst*-1/FGF4 and *bcl*-1) located within the 11q13 amplification unit remains to be determined. Actually, the 11q13 amplicon is a large amplification unit and it is likely that irrelevant genes may be co-amplified with a target gene. Involvement of other potential target genes, mapping the q13 region of chromosome 11, in the genesis and/or progression of a subset of human cancer is effectively strengthened by the fact that neither *hst*-1/FGF4 nor *int* 2/FGF3 genes were consistently found expressed in tumours that contain amplification [15]. In contrast, the *Prad*1 gene, which is linked to the *bcl*-1 locus [28] and is identical to the cyclin D1 gene encoding a cell-cycle regulatory protein, is supposed more relevant, since it was found overexpressed together with 11q13 DNA amplification in breast tumours and head and neck SCC cell lines [17]. In our study, all tumours with *hst*-1 amplification contained amplification of the *Prad*1 gene; moreover 2 cases displayed amplification of the *hst*-1/FGF4 and of the *Prad*1 genes in the absence of *bcl*-1 gene amplification. This suggests that, in head and neck tumours like in breast tumours, the *Prad*1 gene is more likely to be the key feature in the amplification unit than the *bcl*-1 locus, provided it is expressed. It seems of interest to define the limit of the 11q13 amplicon specifically involved in head and neck SCC.

Otherwise, *hst*-1(FGF4)/*int* 2(FGF3) gene coamplification was reported at a high frequency (40–50%) in oesophageal carcinomas [12], a cancer of the squamous cell type related to the head and neck SCC since similar risk factors, like tobacco and alcohol, appear also to be aetiologically involved.

Interestingly, it has been shown that the frequency of fragile sites is markedly higher in peripheral blood lymphocytes from smokers compared to those from non-smokers [38]. 11q13 and the *bcl*-1 locus particularly, is one of these sites. Moreover, the whole upper aerodigestive mucosa is exposed to the same carcinogens leading to the so-called "field cancerisation" which is reflected clinically by a high incidence of simultaneous multiple neoplasia or second primary cancer. In our series, more than 80% of cases presenting simultaneous primary tumours display *hst*-1/*bcl*-1 gene coamplification (15/18 cases) in the principal tumour sample and the same amplification pattern was detected in the two synchronous tumours of a patient. Mutagens included in tobacco/alcohol may play a role in the relative high rate and similarity of pattern of 11q13 amplification observed in the various head and neck and oesophageal carcinomas.

The amplification of *hst*-1/FGF4 and *bcl*-1 genes may be the fortuitous hallmark of unknown essential gene(s) within the 11q13 amplicon. Our results provide support for the involvement of genes mapping 11q13 in the progression of head and neck cancer. Consequently, 11q13 amplification could provide additional useful information for identifying a subset of agressive tumours with high recurrence potential. This has been suggested by Somers *et al.* [32] which described a possible relation between *int* 2/FGF3 gene amplification and tumour recurrence (21 cases tested). Thus, the significance of *hst*-1(FGF4)/*bcl*-1 coamplification as a prognostic factor in head and neck SCC seems promising and further analyses that need a sufficient follow-up time are currently in progress in our prospective study.

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