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Coamplification of the *hst* and *bcl*-1 Oncogenes in Advanced Squamous Cell Carcinomas of the Head and Neck

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Squamous cell carcinomas of the head and neck from 40 untreated patients were analysed for rearranged or amplified proto-oncogenes by Southern blot hybridisation. The *bcl*-1 and the *hst* genes were coamplified 8–32-fold in 5 patients (12.5%). Only males with stage III and IV disease showed coamplification of these oncogenes. Northern blot analysis of the positive samples did not show expression of *bcl*-1 or *hst* genes. In contrast, a third oncogene located on chromosome 11 (Ha-*ras*-1) was not amplified in these tumours. Disease development was observed in all cases over a minimum period of 3 years. Survival of the patients with coamplification of *hst*/*bcl*-1 seemed to be shorter than of those with stage III and IV disease without amplification. This difference was not significant probably due to the small number of investigated patients.
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INTRODUCTION

LIMITED INFORMATION is available on the involvement and role of oncogene amplification in the development and prognosis of head and neck squamous cell carcinomas (SCC). In contrast to other human tumours, statistically significant correlations between amplified oncogenes and clinicopathological parameters of head and neck cancer are not known [1, 2].

Recently some authors have described an uncommonly high rate of gene amplification of two oncogenes, *bcl*-1 and *int*-2, which have been shown to share the same gene locus [3, 4]. *Bcl*-1 has been recognised as the breakpoint of a chromosome translocation in various B-cell malignancies. The breakpoint has been cloned and the region with a putative oncogene has been named bcl-1 (B-cell lymphoma/leukaemia-1) [5]. The *int*-2 gene is known as a member of the family of fibroblast growth factor-related genes. These genes are suggested as important factors in cell growth modulation, because they are active in the early embryogenesis but not in normal adult tissues [6]. Furthermore, a third oncogene, *hst*, is located at the same locus. It shows coamplification with the *int*-2 oncogene in some human cancers [7].

Therefore the question arises whether mutations of this locus play a role in head and neck carcinomas. We analysed fresh DNA and RNA samples from untreated squamous cell carcinomas of the head and neck for amplification, rearrangement and expression of the *bcl*-1 locus and the *hst* gene.

The results were correlated with stage of disease, primary site, differentiation pattern of tumour tissue and the survival data of the patients with a minimum follow-up of 3 years. Furthermore, we analysed the tobacco consumption of our patients since the mentioned gene locus is known as a fragile site for chromosomal mutations in the chromosomes of smokers [8].

PATIENTS AND METHODS

Patients and sample material

Between August 1986 and December 1987 tissue samples of 40 untreated patients with squamous cell carcinomas of the head and neck were collected during panendoscopy or surgery, frozen immediately in liquid nitrogen, and stored at −70°C. In 5 cases additionally normal, adjacent mucosa tissue was available and also underwent investigation. In 3 patients tumour tissue not only from the primary site but also from a lymph node metastasis was available. Granulocytes of healthy donors and normal mucosa tissue of some patients were used as control samples.

The stage of disease was determined according to the TNM-system of the UICC (3rd edition).

The smoking history data collected included the form of tobacco used, starting and stopping age and the average consumption over the previous 12 months.

Isolation of DNA from tissue

After confirmation of the histological diagnosis of the frozen tissue by cryostat sectioning, the tissues were minced, homogenised, and incubated in 10 mmol/l Tris (pH 7.5), 10 mmol/l EDTA, 200 μg/ml proteinase K and 0.2% sodium dodecyl sulphate (SDS) at 37°C for 6 h. The DNA was then extracted with phenol/chloroform/isoamylalcohol, precipated in ethanol, dissolved in 10 mmol/l Tris (pH 7.5) and 1 mmol/l EDTA and stored at 4°C. DNA concentration was measured by absorbance at 260 nm.

Isolation of RNA from tissue

Tissues were minced and homogenised in 4 mol/l guanidinium isothiocyanate containing 25 mmol/l Na citrate, 0.1 mol/l β-mercaptoethanol, 0.5% *N*-lauroylsarcosine, 0.1% antifoam A. Lysates were layered over a 2.5 ml 5.7 mol/l CsCl-EDTA cushion and centrifuged in a SW41 rotor for 16 h [9].

The RNA pellet was dissolved in 10 mmol/l Tris (pH 7.5), 1 mmol/l EDTA. RNA was extracted twice each with phenol/chloroform/isoamylalcohol and chloroform/isoamylalcohol, precipitated in ethanol, and stored at −70°C. RNA concentration was measured by absorbance at 260 nm.

Southern blot analysis

High molecular weight DNA was digested with the appropriate restriction enzyme (Boehringer Mannheim, F.R.G.). Individual lanes of a 1% agarose gel were loaded with 10 μg of digested DNA. Following electrophoresis the fragments were subsequently denatured, and transferred to nytran membranes.

Northern blot analysis

RNA was denatured for 30 min at 65°C and loaded into 1% agarose gels containing 1 × MOPS [20 mmol/l 3-(*N*-morpholine) propane-sulphonic acid), 5 mmol/l Na-acetate, 1 mmol/l EDTA pH 7.0 and 0.23 mol/l formaldehyde. Following electrophoresis the RNA was transferred to nytran membranes by blotting overnight in 20 × SSC (1 × SSC = 0.15 mol/l NaCl, 0.015 mol/l Na-citrate pH 7.0).

Hybridisation

Filters were prehybridised for 3–4 h in 4 × SSC, 0.1% Denhardt's solution, 0.05 mol/l Na-phosphate, denatured and sonicated salmon sperm DNA (500 μg/ml), 0.1% SDS and 50% formamide. DNA probes were labelled by random hexanucleotide priming [10] to specific activities of 1–2 × 10^9 cpm/μg DNA. Hybridisation was performed overnight at 42°C with the labelled denatured probes (Table 1) as described [11, 12]. To confirm uniform DNA loading the single copy gene β1-interferon (β1-IF) was used as a control probe. After hybridisation the filters were washed at high stringency (0.1 × SSC, 0.1% SDS at either 50°C or 62°C). Labelled bands were detected by exposing the filters to Kodak XAR-5 T films at −70°C for 3–14 days with intensifying screens. The intensity of the hybridisation signals was determined densitometrically (2202 Ultrascan, LKB). Finally, the degree of amplification was estimated by slot-blot hybridisation of serially diluted DNA from tumours and normal granulocytes.

The RNA-blots were washed in 2 × SSC, dried and baked for 2 h at 80°C. Transcript sizes were estimated relative to the migration of 28s (5.0 kb) and 18s (2.0 kb) rRNA bands in ethidium bromide stained gels. To control for variable RNA loading, the filters were rehybridised with an actin-specific probe.

Probe-stripping was achieved by washing in 5 mmol/l Tris (pH 7.5), 0.1 mmol/l EDTA, 0.1% Denhardt's for 1 h at 75°C.

Probes

The following probes were used in this study: *bcl*-1 2.1 kb *Bam*HI [5], *hst* 0.6 kb, *Hind*III [13], Ha-*ras*1 6.6 kb *Bam*HI [14], interferon β1 1.9 kb *Eco*RI [15], actin 3.6 kb *Hind*III [16].

Statistical methods

Statistical analysis of survival was performed by a product limit estimation of survival [17] using the BMDP statistical software package [18]. We used the date from onset of specific therapy as time origin. The event analysed is the death of a patient, all other survival times are treated as (right-) censored. Patients were observed for at least 36 months.

Equality of distributions of survival times across groups were tested by a non-parametric linear rank test according to Breslow [19]. The analysis of statistical dependency between extend of nicotine abuse and oncogene amplification was done by Fisher's exact test (one-tailed) [18].

RESULTS

Southern blot analysis

Digested DNA was analysed by Southern blot analysis using the *bcl*-1, *hst*, Ha-*ras*-1 and β1-IF. Five out of 40 (12.5%) SCC DNA showed amplification of the *hst* and *bcl*-1 gene compared

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Table 1. *Clinicopathological data of patients and degree of* bcl-1 *and* hst *amplification in their primary tumours*

No.	Age/sex (years)	Site	TNM/stage	Differentiation grade	Amplification		Status (month)
					bcl-1	*hst*	
8	58/F	Tongue	T1N0M0/I	p	—	—	
27	52/M	Oropharynx	T1N0M0	w	—	—	
41	64/M	Larynx	T1N0M0	m	—	—	
45	61/F	Larynx	T1N0M0	m	—	—	
3	64/F	Tongue	T2N0M0/II	w	—	—	
4	53/F	Oropharynx	T2N0M0	m	—	—	
6	48/M	Nasopharynx	T2N0M0	p	—	—	
9	74/M	Oropharynx	T2N0M0	p	—	—	
10	67/F	Larynx	T2N0M0	w	—	—	
28	55/M	Oropharynx	T2N0M0	m	—	—	
37	48/M	Larynx	T2N0M0	p	—	—	
11	67/M	Oropharynx	T3N0M0/III	m	—	—	D (19)
21	47/M	Hypopharynx	T3N0M0	m	—	—	DWD (9)
31	65/F	Nasopharynx	T3N0M0	p	—	—	A (40)
42	65/M	Larynx	T3N0M0	m	—	—	DFS (36)
14	66/M	Larynx	T3N0M0	m	16 ×	16 ×	D (18)
23	68/M	Oropharynx	T3N1M0	m	—	—	DWD (6)
2	51/M	CUP	T×N3M0/IV	w	—	—	D (12)
22	56/F	CUP	T×N3M0	m	—	—	DFS (36)
43	42/M	Oropharynx	T1N3M0	m	—	—	DFS (38)
1	33/M	Nasopharynx	T2N2M0	p	—	—	DFS (50)
35	72/M	Oropharynx	T2N3M0	m	—	—	DWD (18)
32	42/M	Nasopharynx	T3N2M0	p	—	—	DFS (44)
36	49/M	Hypopharynx	T3N2M0	m	—	—	D (24)
20	54/M	Oropharynx	T3N3M0 +	m	—	—	DFS (40)
33	63/M	Oropharynx	T3N3M0	m	—	—	A (36)
40	43/M	Hypopharynx	T2N3M0	w	8 ×	8 ×	D (24)
12	47/F	Larynx	T4N0M0	m	—	—	DWD (21)
15	52/F	Nasopharynx	T4N0M0	m	—	—	D (20)
30	55/F	Hypopharynx	T4N0M0	m	—	—	D (16)
16	55/M	Larynx	T4N1M0 +	m	—	—	DFS (51)
25	56/M	Oropharynx	T4N1M0	w	32 ×	32 ×	D (6)
34	45/M	Oropharynx	T4N1M0	m	16 ×	16 ×	D (22)
5	52/M	Hypopharynx	T4N3M0	m	—	—	D (15)
7	53/M	Oropharynx	T4N3M0	m	—	—	D (21)
17	49/M	Hypopharynx	T4N3M0	p	—	—	D (6)
26	50/M	Oropharynx	T4N3M0 +	m	—	—	D (6)
38	51/M	Hypopharynx	T4N3M0	p	—	—	D (12)
39	42/M	Oropharynx	T4N3M0	m	—	—	D (12)
44	51/M	Hypopharynx	T4N3M0	m	16 ×	16 ×	D (12)

M = Male, F = female. Differentiation grade: w = well, m = moderately, p = poorly. Status: A = Alive with tumour, D = dead with tumour, DFS = disease free survival, DWD = dead without disease. + = lymph node metastasis and primary tumour were investigated.

with normal tissue and granulocyte DNA. All 5 cases showed coamplification of both *bcl*-1 and *hst* genes. Neither a rearrangement of *hst* nor of *bcl*-1 was detectable. An example for coamplification of *hst* and *bcl*-1 is shown in Fig. 1a. The signal of the single copy gene β1-IF indicated that each lane contained comparable amounts of total DNA (Fig. 1b). Chromosome 11 multiplication was excluded because the other chromosome 11 specific proto-oncogene (Ha-*ras*) did not show amplification in any of these 5 cases (data not shown). Furthermore, no Ha-*ras* amplification was found in any of the other tumours when comparing the intensity of the signal with the relative amount of DNA at each lane and the signal of normal mucosa and granulocytes. The results are summarised in Table 1.

Laser densitometry and slot blot analysis of amplification

The presumption of coamplification of *bcl*-1 and *hst* was confirmed by densitometric scanning (data not shown). The degree of gene amplification was investigated by serial dilution and slot blot hybridisation of each DNA sample (Fig. 2).

The degree of amplification (8–32-fold) was the same for *bcl*-1 as for *hst* (Table 1). This suggests amplification of a whole amplification unit ('amplicon') including *bcl*-1 and *hst*.

Northern blot analysis

Gene amplification may be accompanied by enhanced gene expression. However, in spite the presence of intact RNA (confirmed by additional hybridisation with an actin-specific

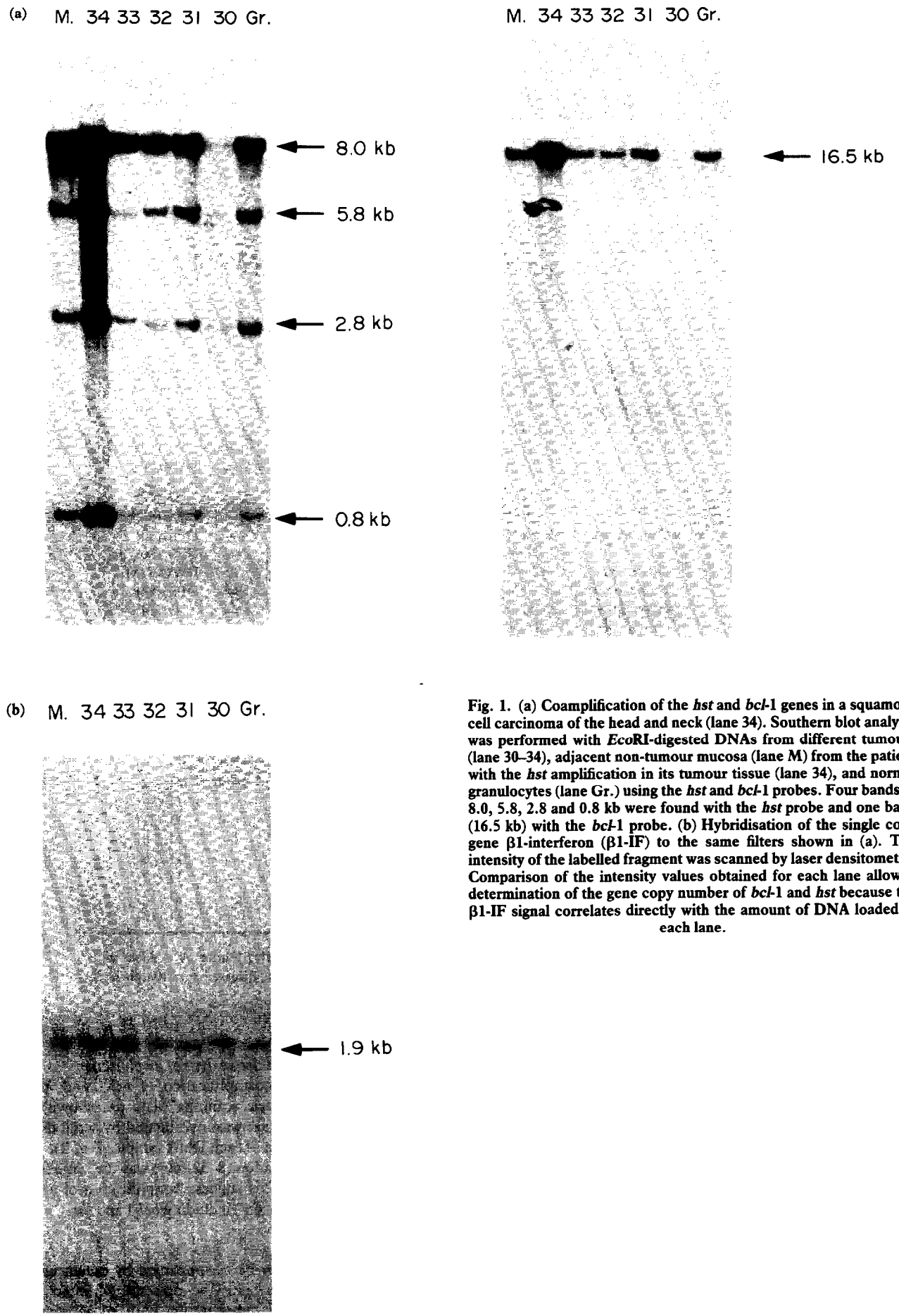

Fig. 1. (a) Coamplification of the *hst* and *bcl*-1 genes in a squamous cell carcinoma of the head and neck (lane 34). Southern blot analysis was performed with *Eco*RI-digested DNAs from different tumours (lane 30–34), adjacent non-tumour mucosa (lane M) from the patient with the *hst* amplification in its tumour tissue (lane 34), and normal granulocytes (lane Gr.) using the *hst* and *bcl*-1 probes. Four bands of 8.0, 5.8, 2.8 and 0.8 kb were found with the *hst* probe and one band (16.5 kb) with the *bcl*-1 probe. (b) Hybridisation of the single copy gene β1-interferon (β1-IF) to the same filters shown in (a). The intensity of the labelled fragment was scanned by laser densitometry. Comparison of the intensity values obtained for each lane allowed determination of the gene copy number of *bcl*-1 and *hst* because the β1-IF signal correlates directly with the amount of DNA loaded to each lane.

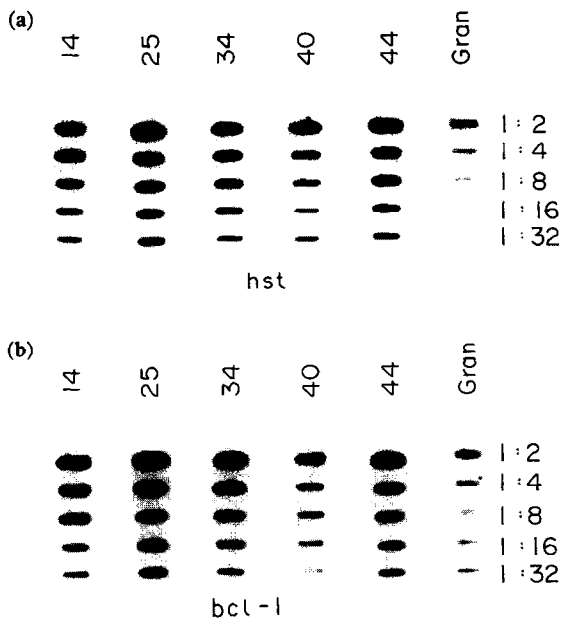

Fig. 2. Slot blot hybridisation of serial diluted DNA amounts from all tumours (14,25,34,40,44) with *hst* (a) and *bcl*-1 (b) amplification and from normal granulocytes (Gran.) as a control. The degree of amplification of *hst* and *bcl*-1 was always the same, providing further evidence for coamplification of both genes. Cross-hybridisation of slot blots was excluded by simultaneous hybridisation of Southern-blotted granulocytes DNA showing the appropriate band (data not shown).

probe), none of the tumours with coamplification of *bcl*-1 and *hst* demonstrated any expression of these two genes.

Relationship between coamplification and clinicopathological data

Coamplification was found in 4 patients with stage IV disease and 1 patient with stage III, but not in patients with stage I or II disease. Only male patients showed the coamplification. There was no correlation between coamplification and primary tumour site or histological types of SCC (Table 1).

The analysis of overall patient survival (stage III and IV disease) showed no statistical significance for rejecting the hypothesis of equal distributions ($P = 0.334$). Because of the small number of patients under observation—especially in one group—this result must be interpreted carefully. However, the survival curves for both groups indicate that patients with coamplifiation of *bcl*-1 and *hst* may have a poorer prognosis than patients without amplification (Table 1 and Fig. 3).

Tobacco consumption and coamplification

Because coamplification of *hst* and *bcl*-1 was only found in patients with stage III and IV disease, the tobacco consumption of this subgroup is considered. 3 patients are non-smokers. 26 of 29 patients (90%) have a smoking history of 20 years or more during their adult life. 2 of these 26 patients stopped smoking in recent years ('ex-smokers'). All other patients were smoking up to the interview. Coamplification was not seen in non-smokers nor in ex-smokers. Analysis of tobacco consumption indicated that coamplification and abuse of nicotine (defined as smoking more than 20 cigarettes per day over more than 20 years) are related, at least statistically ($P = 0.0521$). All males with coamplification are current smokers who have smoked more than 20 cigarettes per day over at least 20 years (Table 2).

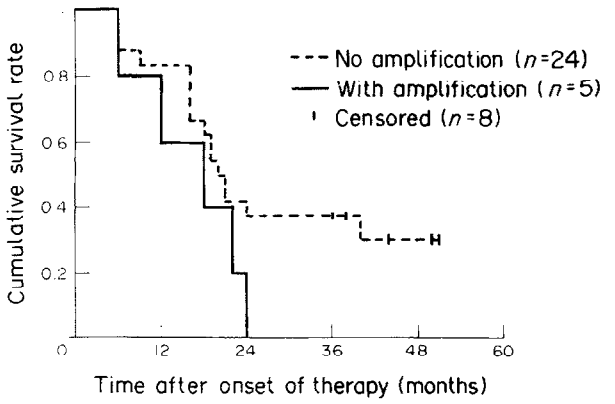

Fig. 3. Survival curves are drawn up using the Kaplan–Meier product limit estimates [17]. For the purpose of these survival curves amplification is defined as a value of at least 4-fold or more than the hybridisation signal of the *hst* and *bcl*-1 genes found in normal tissue.

DISCUSSION

Recently Berenson first described the amplification of the *bcl*-1 locus without an expression of *bcl*-1 in head and neck squamous cell carcinomas [3]. This was the first report on abnormalities of

Table 2. Degree of bcl-1 *and* hst *amplification and smoking history*

Patient No.	Amplification *bcl*-1	Amplification *hst*	Smoking years	Average cigarettes/day smoked during last 12 months
Non-smokers				
1	—	—		
31	—	—		
32	—	—		
Ex-smokers				
26	—	—	30	
30	—	—	25	
Smokers				
< 20 cigarettes/day				
20	—	—	35	< 10
5	—	—	30	10–20
11	—	—	45	10–20
15	—	—	20	10–20
16	—	—	40	10–20
23	—	—	40	10–20
39	—	—	25	10–20
> 20 cigarettes/day				
14	16 ×	16 ×	40	20–30
17	—	—	30	20–30
22	—	—	25	20–30
33	—	—	30	20–30
35	—	—	50	20–30
38	—	—	30	20–30
42	—	—	45	20–30
43	—	—	25	20–30
2	—	—	30	30–40
40	8 ×	8 ×	20	30–40
44	16 ×	16 ×	35	30–40
7	—	—	35	40–60
12	—	—	30	40–60
21	—	—	20	40–60
25	32 ×	32 ×	35	40–60
34	16 ×	16 ×	25	40–60
36	—	—	30	40–60

the *bcl*-1 locus in solid tumours. More recently the finding of a coamplification of the *bcl*-1 locus, *hst* and *int*-2 in human breast cancer suggested a common amplification unit on the chromosome 11 band q13 entailing all three loci ('amplicon') [20]. Finally, in oesophageal carcinomas a high incidence of coamplification of *hst*-1 and *int*-2 genes was described [21].

Hst is a transforming gene that has been isolated from two human stomach cancers using an NIH/3T3 transfection assay [22]. It encodes a heparin binding growth factor for human vascular endothelial cells [23]. The *int*-2 gene belongs to the same gene family as the *hst* gene, which encodes acidic and basic fibroblast growth factors (FGF) [6]. Both groups of FGF are highly angiogenic and mitogenic for a wide variety of cell types. Therefore, one has speculated that the need for vascularisation during tumour development provides the selective pressure for tumour cells containing an *int*-2 or *hst* amplification.

In our study five out of 40 (12.5%) squamous cell carcinoma DNA samples showed 8–32-fold amplification of the *hst* proto-oncogene. All these DNA samples showed coamplification of the *bcl*-1 locus. The degree of amplification, estimated by slot-blot analysis, was the same for *hst* and the *bcl*-1 locus. This provides further evidence for an amplicon which includes *bcl*-1 and *hst* in squamous cell carcinomas of the head and neck.

Furthermore, we found no amplification of Ha-*ras*, another putative oncogene on chromosome 11. This indicates that coamplification of the *hst* and *bcl*-1 genes does not imply an increased number of chromosomes 11 in head and neck cancer.

In concurrence with previously mentioned studies [3, 21], we could not demonstrate either *bcl*-1 or *hst* gene expression by northern blot analysis in our tumour samples. Up to now, only single cases of amplification with expression of the mentioned genes are known [24]. Therefore, the search for additional genes within this amplicon that may be more critical to tumour growth was intensified in the past [25]. Recently, Schuuring and colleagues described an amplification and overexpression of two further proto-oncogenes—PRAD-1 and *ems*-1—belonging to the 11q13 locus [26].

Correlating our results with the clinicopathological data of the patients, we made the following observations: coamplification of *hst* and *bcl*-1 was only found in male patients with advanced stage of disease. Although both findings must be interpreted with care because of the small number of patients, they confirm the recently published data in human oesophageal carcinomas [21].

Coamplification did not correlate with primary tumour site or histological type of SCC. The latter is in contrast to the published data in squamous cell carcinoma of the lung with a more frequent amplification of the *bcl*-1 locus in poorly differentiated tumours [27].

It has been proposed that amplification of the mentioned amplicon could be a marker for a poorer prognosis in patients with advanced stage of disease [4, 28]. This is the first study in head and neck cancer, which correlates long-term survival data with *hst* and *bcl*-1 abnormalities. No patient with coamplification was alive within 2 years after starting therapy and all of these patients died of their head and neck cancer. However, results were statistically not significant owing to the small number of patients. Therefore, the clinical value of this marker should be investigated in more patients.

Another interesting finding was the positive correlation between high tobacco consumption and coamplification. This confirms the preliminary data of Berenson and his colleagues [3]. Recently, it has been shown that peripheral blood lymphocytes of smokers have a higher frequency of specific chromosomal fragile sites than lymphocytes of non-smokers. One of the specific fragile sites is the band q13 on chromosome 11 with the mentioned amplicon [8]. Because tobacco abuse is considered to be the most important acquired cause of head and neck cancer, it might be possible that mutagens found in cigarette smoke play a role in the relative high rate of amplification of 11q13 in head and neck cancer.

Up to this day the significance of the amplification of the mentioned amplicon for the development of head and neck cancer remains unclear. Further studies are necessary to elucidate role of abnormalities on chromosome 11 band q13 in human malignancies.

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Derivation and Characterisation of a Mouse Tumour Cell Line with Acquired Resistance to Cyclosporin A

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Cyclosporin A (CsA) is an effective modifier of multidrug resistance. We have studied (a) the possibility that cells grown in increasing concentrations of CsA acquire cellular resistance to the agent and, (b) whether such cells have a multidrug resistant phenotype. Sublines of the EMT6 mouse tumour cell line were developed which were able to grow in 75 and 200 μg/ml of CsA, respectively. The resistant sublines grew slowly in the presence of CsA but reverted to control growth rates, whilst maintaining resistance, when the drug was removed. P-glycoprotein (Pgp) was not detectable in the resistant sublines by immunocytochemistry. The CsA-resistant cells were not cross-resistant to doxorubicin or vincristine but showed a clear degree of cross-resistance to the calcium transport blocker, verapamil. Cellular accumulation of both [^{3}H]CsA and [^{3}H]daunorubicin was significantly increased in the EMT6/CsA200R subline compared with the parent line. In the EMT6 parent line, which expresses very low levels of Pgp, 10–30-fold sensitisation to doxorubicin may be achieved using 0.1–5 μg/ml of CsA. Similar sensitisation by CsA was also seen in the CsA-resistant sublines.
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INTRODUCTION

A MAJOR FACTOR limiting the effectiveness of cancer chemotherapy is the emergence of drug-resistant cells. Several mechanisms can lead to cross-resistance to broad groups of agents, one of which is known as classical multidrug resistance (MDR) [1, 2]. This mechanism involves overexpression of P-glycoprotein, a membrane transport glycoprotein thought to act as a drug efflux "pump" [1, 2]. A number of chemical agents, including verapamil (VRP) and cyclosporin A (CsA) have been shown to act as modifiers of this form of resistance in that they are able, at least partially, to restore drug sensitivity to MDR cells [3–5]. Both VRP and CsA have been shown to bind to P-glycoprotein and to compete for binding with drugs involved in the MDR phenotype [6–8]. In addition, both agents have shown to accumulate to a lesser extent in some MDR cells than in wild-type drug sensitive cells [9, 10]. As agents involved in the MDR phenotype are generally able themselves to induce such a phenotype, we are interested in the question of whether such resistance modifiers possess this property. We have previously described the derivation and characterisation of a mouse tumour cell line with acquired resistance to VRP [11]. In this paper we describe the independent derivation and characterisation of CsA-resistant subline.

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MATERIALS AND METHODS

Cell lines

The EMT6/Ca/VJAC mouse mammary carcinosarcoma cell line has previously been described [12, 13], as have the resistant sublines EMT6/AR 1.0 [14] and EMT6/VRP [11].

All cell lines were grown in Eagle's minimum essential medium (MEM) with Earle's salts supplemented with glutamine (0.5 mmol/l), penicillin (100 U/ml), streptomycin (100 μg/ml) and 20% new-born calf serum (NBCS) (Life Technologies).