Pergamon

Oral Oncol, Eur J Cancer, Vol. 31B, No. 6, pp. 384–391, 1995

0964-1955/95 $9.50 + 0.00

0964-1955(95)00045-3

Differential *c-myc*, *c-jun*, *c-raf* and *p53* Expression in Squamous Cell Carcinoma of the Head and Neck: Implication in Drug and Radioresistance

C. Riva, J.-P. Lavieille, E. Reyt, E. Brambilla, J. Lunardi and C. Brambilla

The expression of oncogenes *c-myc*, *c-jun* and *c-raf* and tumour suppressor gene *p53* was assessed by northern blot analysis of 42 tumours and p53 protein expression by immunohistochemistry on paraffin-embedded sections from 36 specimens of squamous cell carcinoma of the head and neck (SCCHN) obtained before therapy. Of the 42 tumours, 89, 100 and 100% expressed *c-myc*, *c-jun* and *c-raf* oncogenes, respectively. These oncogene expressions did not correlate with sex, age or clinical stage of the disease. However, an association was found between low *c-myc* expression ($P=0.0001$) and high *c-jun* expression ($P=0.0001$) and absence of tumoral response to neoadjuvant chemotherapy. On the other hand, *c-raf* overexpression was observed in patients resistant to radiation therapy ($P=0.0494$). Forty-two per cent of the tumours showed p53 protein overexpression, which did not correlate with any clinical parameter. This p53 protein overexpression was associated with high *p53* mRNA levels (REL) ($P=0.0223$). A correlation was found between increased *c-myc* RNA expression and lack of p53 protein expression ($P=0.0407$). In addition, a lack of p53 protein expression was indicative of tumour relapse ($P=0.05$). None of these biological parameters were associated with disease-free survival (Cox-Mantel test). In conclusion, the overexpression of *c-myc*, *c-jun* and *c-raf* may be independently associated to tumoral response to chemotherapy or radiotherapy, or to tumour relapse, but fail to predict long-term survival.

Keywords: oncogenes, p53 protein, squamous cell carcinoma, head and neck

Oral Oncol, Eur J Cancer, Vol. 31B, No. 6, pp. 384–391, 1995.

INTRODUCTION

SQUAMOUS CELL carcinoma (SCC) constitute at least 90% of all upper aerodigestive tract malignancies [1]. The clinical observation that patients with head and neck cancer in comparable stages may run different clinical courses and may respond differently to similar treatments has yet to be adequately understood. Although clinical outcome is influenced by stage of disease and histomorphological features, currently used prognostic factors remain subjective and poorly standardised. While studies to investigate and delineate the regulatory control mechanisms of head and neck malignant tumours have been undertaken, the majority of the studies centre on the implication of gene amplification and overexpression in prognosis [2–4]. However, mechanisms controlling proliferation and neoplastic behaviour after treatment of SCCs of the upper digestive tract remain to be understood.

In this study, we have investigated the expression of *c-myc*, *c-jun* and *c-raf* oncogenes in 42 small cell carcinomas of the head and neck (SCCHN) of different clinical stages to determine whether there was an association between the profile of expression and tumour stage. The *c-myc* gene codes for a nuclear protein p62 which is involved in cell growth, differentiation and programmed cell death [5]. The product of the *c-jun* oncogene is postulated to have DNA binding activity. The product of the *c-raf* oncogene is a cytoplasmic serine-threonine kinase and has been shown to be implicated in drug and radiation resistance [6, 7]. These oncogenes were selected in this study to determine if a pattern of co-activation of cell membrane associated cytoplasmic and/or nuclear oncogenes could be observed in different tumour stages and was correlated to any clinicopathological parameters, chemoresistance, radioresistance and survival.

Correspondence to C. Riva.
C. Riva, J.-P. Lavieille and C. Brambilla are at the Lung and Airways Cancer Research Group, Albert Bonniot Institute, Faculty of Medicine, La Tronche, France; E. Reyt is at the Head and Neck Department; E. Brambilla is at the Cellular Pathology Department; J. Lunardi is at the ADN Biochemistry Department, University Hospital, Grenoble, France.
Received 16 May 1995; provisionally accepted 30 May 1995; revised manuscript received 4 July 1995.

Functional inactivation of the tumour suppressor gene *p53* is such a common event in many human cancers that it has been suggested as a tumour specific marker. A number of studies investigated correlations between *p53* status and the progression of different solid tumours of the head and neck [8]. Following DNA damage, the increase of p53 protein level leads to an arrest of cell division in G1 phase [9]. This arrest allows the activation of a DNA repair system. In some cases, DNA repair is not efficient, and a sufficient level of wild-type p53 protein may induce apoptosis (programmed cell death) [9]. Since chemotherapy or radiotherapy lead to DNA damages, *p53* status investigation should be interesting in order to define the pretreatment profile of p53 protein in tumour cells, according to the response to treatment. Our goal was to investigate whether the determination of *p53* status has an implication in tumour progression, resistance and survival of SCC of the head and neck.

In the current study, we report our results for *c-myc*, *c-jun*, *c-raf* and *p53* expression and co-expression in SSCHN, and have correlated the results with the already known clinical and pathological prognostic factors.

MATERIALS AND METHODS

Tumour tissues

Tumour tissues of oral SCC were obtained from 42 patients treated at the ENT Unit, University Hospital, Grenoble, France (Table 1). Tumour specimens were obtained at the time of diagnosis, before any treatment, from the excised material. Tissues with necrosis, haemorrhage or calcification

Table 1. Clinical characteristics of patients

Case	Age years (sex)	Primary site	Differentiation	TNM staging	Treatment*	Clinical response†	Prognosis (mo)	Outcome‡
1	51 (M)	Pharynx	Well	$T_2N_{2B}M_0$	CT	PR	32	ANED
2	73 (M)	Pharynx	Mod.	$T_3N_1M_0$	CT	PR	15	DED
3	52 (M)	Pharynx	Well	$T_3N_0M_0$	CT	PR	26	DED
4	36 (M)	Oral cavity	Well	$T_3N_1M_0$	CT	ST	30	DED
5	42 (M)	Pharynx	Well	$T_2N_0M_0$	CT	CR	25	ANED
6	49 (F)	Pharynx	Well	$T_3N_{2A}M_0$	CT	CR	22	ANED
7	61 (M)	Pharynx	Mod.	$T_3N_0M_0$	CT	PR	36	AED
8	57 (M)	Pharynx	Well	$T_3N_0M_0$	CT	ST	35	DED
9	55 (F)	Pharynx	Poorly	$T_4N_{2C}M_0$	CT	ST	6	DED
10	59 (M)	Pharynx	Well	$T_3N_{2C}M_0$	CT	ST	7	DED
11	61 (M)	Pharynx	Well	$T_3N_0M_1$	CT	PR	36	AED
12	61 (M)	Pharynx	Well	$T_2N_0M_1$	CT	CR	35	ANED
13	45 (M)	Tongue	Well	$T_2N_0M_0$	CT	PD	5	DED
14	46 (M)	Pharynx	Well	$T_3N_{2B}M_0$	CT	ST	33	AED
15	47 (M)	Adenopathy	Well	$T_0N_3M_0$	CT	PR	12	DED
16	50 (M)	Pharynx	Poorly	$T_3N_0M_0$	CT	CR	15	DED
17	54 (M)	Pharynx	Well	$T_3N_1M_0$	CT	PR	37	ANED
18	69 (M)	Pharynx	Well	$T_3N_0M_0$	CT	PR	19	DED
19	42 (M)	Pharynx	Poorly	$T_1N_3M_1$	CT	PD	11	DED
20	71 (M)	Pharynx	Mod.	$T_3N_{2C}M_1$	CT	PR	33	DED
21	65 (M)	Oral cavity	Well	$T_3N_0M_0$	CT	CR	5	ANED
22	62 (M)	Pharynx	Poorly	$T_2N_0M_0$	CT	CR	23	ANED
23	57 (M)	Pharynx	Well	$T_2N_{2B}M_0$	CT	CR	30	ANED
24	63 (M)	Larynx	Well	$T_4N_{2C}M_0$	CT	CR	20	DED
25	59 (M)	Pharynx	Mod.	$T_1N_0M_0$	RT	CR	19	ANED
26	63 (M)	Pharynx	Poorly	$T_4N_{2C}M_1$	RT	PD	6	DED
27	48 (M)	Pharynx	Well	$T_4N_{2C}M_0$	RT	PD	5	DED
28	57 (M)	Pharynx	Well	$T_2N_1M_0$	RT	CR	37	DED
29	74 (M)	Pharynx	Well	$T_3N_0M_0$	RT	PD	10	DED
30	71 (M)	Pharynx	Well	$T_3N_{2C}M_0$	RT	ST	21	ANED
31	63 (M)	Pharynx	Well	$T_2N_0M_0$	RT	CR	27	ANED
32	61 (M)	Pharynx	Well	$T_2N_{2C}M_0$	RT	PD	15	AED
33	61 (M)	Oesophagus	Well	$T_3N_0M_0$	RT	PR	1	DED
34	68 (M)	Pharynx	Well	$T_2N_{1A}M_0$	RT	CR	28	ANED
35	39 (M)	Pharynx	Poorly	$T_1N_0M_0$	RT	CR	33	ANED
36	63 (M)	Pharynx	Well	$T_1N_1M_0$	RT	CR	29	ANED
37	77 (M)	Pharynx	Well	$T_3N_0M_1$	RT	CR	20	DED
38	81 (M)	Oral cavity	Well	$T_1N_0M_0$	RT	PR	2	AED
39	55 (M)	Pharynx	Mod.	$T_3N_{2C}M_0$	RT	CR	22	ANED
40	73 (M)	Pharynx	Well	$T_2N_1M_0$	RT	CR	12	ANED
41	53 (M)	Pharynx	Well	$T_2N_{2B}M_0$	RT	CR	29	ANED
42	50 (M)	Pharynx	Well	$T_4N_1M_0$	RT	PD	2	AED

M, male; F, Female. *CT, patients treated with chemotherapy; RT, patients treated by radiotherapy. †CR, complete response; PR, partial response; ST, stabilisation; PD, progressive disease. ‡AED, alive with evolutive disease; DED, dead with evolutive disease; ANED, alive non-evolutive disease.

were excluded. Histological confirmation of SCC was made from an adjacent section, and squamous cancer cells comprised 50–100% of the total cell population in all cases. The SCC patients (95% males and 5% females) ranged in age from 36 to 77 years (median 58.1 years). Tumour staging (TNM) was conducted according to the 1987 classification of UICC [10]. The numbers of SCC patients in stages I, II, III and IV were 2, 4, 15 and 21, respectively.

Clinical treatment

24 patients received chemotherapy according to a previously reported regimen [11]. Briefly, a combination of CDDP (100 mg/m^2 i.v. D1) plus 5FU (1 g/m^2 continuous infusion D1–D5) was given monthly for three courses. 18 patients received radiotherapy 60 GY on cervical nodes area and 72 GY on tumoral bed in 35 courses.

Tumoral response was estimated 3 weeks after the completion of chemotherapy and 2 months after the arrest of radiotherapy. Complete response was defined as the complete disappearance of the tumour at the clinical, CT scan and histological examinations of the initial tumoral area. A partial response was noted when a regression of more than 50% of the two largest diameters of the tumour was observed. Stabilisation was a regression of less than 50% of tumoral diameters, and the progressive disease was noted when an increase in size of tumour was observed during treatment. Local recurrence was considered when tumoral regrowth was observed, either after total histological eradication of the tumour by surgery with negative margin of resection, or after complete disappearance of tumours confirmed with histological biopsies after chemotherapy or radiotherapy.

RNA extraction and northern blot analysis

Total RNA was prepared from tumour biopsies by guanidine thiocyanate lysis and the cesium chloride gradient centrifugation method [12]. Approximately 10 µg of total RNA were electrophoresed in denaturing formaldehyde/agarose gels and transferred to hybond N filters or slot blotted on to hybond N filters [12]. Blots were hybridised with oligonucleotide probes at 65°C in 1 M NaCl/50 mM Tris–HCl (pH 7.5)/10% (wt/vol) dextran sulphate/1% (vol/vol) SDS solution containing salmon sperm DNA at 100 µg/ml. Blots were hybridised with a GAPDH probe at 45°C in 5 × SSC/50% (vol/vol) deionised formamide/0.5% SDS/5 × Denhardt's solution containing salmon sperm DNA at 100 µg/ml. The blots were washed with either 2 × SSC/0.1% SDS at 65°C (synthetic probes) or 2 × SCC at 45°C (cDNA probe) and autoradiographed for 48–240 h at −80°C.

Probes

The following probes were used: *c-myc*, *c-raf*, *c-jun* and *p53* from Oncogene Science Inc (Manhasset, New York, U.S.A.). They were all 40 base single-stranded synthetic oligonucleotides (45–50% GC content). High stringency hybridisation and wash procedures as for long DNA probes were used to increase specificity of the probes. These 40mers were end-labelled with (γ32P) ATP using T4 polynucleotide kinase [12].

Hybridisation intensity was measured using a DU-70 spectrophotometer (Beckman). To compare intensity of hybridisation between samples on the same blot, and between samples on different blots, densitometric readings were normalised to the intensity of the GAPDH hybridisation for each level. We determined a relative expression level (REL) as follows

$$\text{REL: } \frac{\text{Densitometric value of oncogene expression}}{\text{Densitometric value of GAPDH expression}}.$$

Immunohistochemistry (IHC)

Thirty-six of the 42 tumours have been analysed for p53 protein expression using immunohistochemistry (IHC), following techniques described previously [13, 14], with four specific antibodies: PAb 1801 (Ab-2, Oncogene Science), DO7 (Dako, Glostrup, Denmark), CM1 (Novocastra Lab., Newcastle, U.K.) and PAb 240 (Ab-3, Oncogene Science) which all recognise most of the mutant and wild-type stabilised p53 forms in denaturing conditions. The threshold for positive nuclear staining was based on the following features: at least 20% of clear positive tumour cells with at least two of the four antibodies tested. Only nuclear staining was taken into consideration. No cytoplasmic staining was consistently observed with all antibodies on the same tumour cells. The term immunophenotype was defined and assigned for the results of p53 immunostaining.

Statistical methods

Associations between categorical variables (TNM stage, pathological grade, presence and expression of oncogene) were investigated using Wilcoxon–Mann–Whitney rank tests. The life tables' techniques based on the Kaplan–Meier procedure were used to estimate univariate differences in relapse and death rates according to these variables. Probability values of less than 0.05 were considered significant. The Cox–Mantel proportional hazards modelling technique was used to identify factors that independently or together significantly influenced overall survival. The overall mortality rate was 45% (19 of 42 patients), and the median follow-up of patients whose tumours had been analysed was 39 months.

RESULTS

Oncogene expression

Clinicopathological features of the patients with SCCHN are shown in Table 1. Nearly all patients had a history of chronic smoking and alcohol use. A total of 42 RNAs from human squamous cell cancers of the oral cavity, larynx and pharynx were studied. Each RNA sample was analysed by northern hybridisation. Filters were serially screened with the different oncogenes, tumour suppressor gene and GAPDH probes. mRNA transcripts of 3.5 kb for *c-raf*, 2.5 kb for *c-myc* and 2.6 kb for *c-jun* were detected in tumour specimens. *c-raf*, *c-myc*, *c-jun* are expressed in 100, 89 and 100% of the tumours, respectively. The mean REL values of expression in this population were 24.6 ± 20.2, 2.99 ± 4.1 and 1.41 ± 0.98 for *c-raf*, *c-myc* and *c-jun*, respectively. There were wide variations in the expression with ranges of 0.60–63.8 for *c-raf* oncogene, 0.23–18.3 for *c-myc* and 0.20–3.8 for *c-jun* oncogene. In the patient population, a higher REL mean value for *c-myc* (5.1) and a lower *c-jun* REL (0.84) were observed in the sensitive group of patients, responding to neoadjuvant chemotherapy compared with patients in the group failing to respond to treatment, with a *c-myc* mean REL of 1.1 and a *c-jun* mean

Table 2. Relationship between mean oncogene expression (REL) and clinical outcome of chemotherapy

Clinical response*	Cases	*c-raf* expression	*c-myc* expression	*c-jun* expression	*p53* expression
CR	7	35.6	6.4	0.88	0.64
PR	9	42.0	4.1	0.81	0.44
Objective response	16	39.2	5.1	0.84	0.54
	(69%)	(15–63.8)	(0.25–18.3)	(0.15–2.0)	(0.10–2.60)
SD	5	19.5	0.90	2.1	0.81
PD	2	15.8	1.5	2.7	0.77
Failure	7	18.5	1.1	2.2	0.80
	(31%)	(10.0–30.0)	(0.29–2.4)	(0.62–3.4)	(0.08–2.07)
Statistical significance†		NS‡	$P=0.0001$	$P=0.0001$	NS

*CR, complete response; PR, partial response; SD, stabilisation disease; PD, progressive disease. †Statistical correlation according to the Mann–Whitney rank test between REL values of objective response patients group (CR plus PR) versus failure patients group (SD plus PD); ‡NS, not significant.

Table 3. Relationship between mean oncogene expression (REL) and clinical outcome of radiotherapy

Clinical response*	Cases	*c-raf* expression	*c-myc* expression	*c-jun* expression	*p53* expression
CR	10	6.2	1.5	1.5	0.65
		(0.60–29.3)	(0.23–2.77)	(0.22–3.30)	(0.12–1.5)
PR	2	25.4	5.9	2.4	2.3
SD	1	29.8	—†	0.51	0.13
PD	5	18.3	1.6	1.5	1.2
Non-response	8	20.9	2.5	1.5	1.4
		(0.89–59.1)	(0.58–5.92)	(0.51–3.40)	(0.13–3.41)
Statistical significance‡		$P=0.0494$	NS§	NS	NS

*CR, complete response; PR, partial response; SD, stabilisation disease; PD, progressive disease; †Not detected; ‡Statistical correlation according to the Wilcoxon test between CR patients versus non-responding patients (PR plus SD plus PD); §NS, not significant.

REL of 2.2. No differences were noted for *c-raf* and *p53* mRNA expression for these two groups (Table 2). In the radioresistant group of patients comprising partial responders and non-responders, *c-raf* expression was increased (REL = 20.9) when compared with the group achieving complete response (REL = 6.2). No differences were observed for the mRNA expression of *c-myc*, *c-jun* and *p53* in this latter group (Table 3).

p53 *analysis*

A mRNA transcript of 2.8 kb was detected in 38 of the 42 tumours. p53 protein accumulation, detected by immunohistochemistry, was observed in 15/36 patients. Of the 15 tumours with positive immunophenotype, 12 exhibited consistent mRNA expression on northern blot analysis with a mean REL of 0.81 ± 0.54 and three tumour samples showed an undetectable level of expression of the usual *p53* transcript. The 20/21 tumours displaying a negative immunophenotype for protein p53 had a REL for *p53* mRNA of 0.53 ± 0.20. We found a direct correlation between overexpression of *p53* mRNA and positive immunophenotype ($P=0.0223$ according to the Mann–Whitney rank test) (Fig. 1).

Statistical correlations

There were no statistically significant associations between the expression of any of these oncogenes (*c-myc*, *c-raf* and

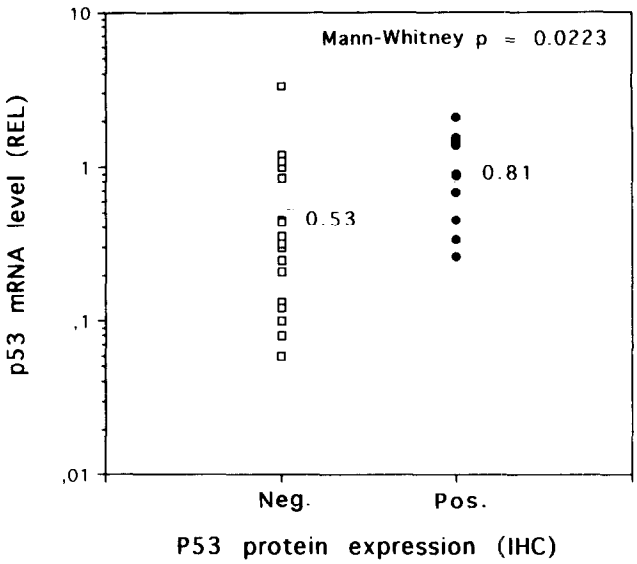

Fig. 1. Correlation between p53 protein expression measured by immunohistochemistry (IHC) noted as positive (pos.) or negative (neg.) as described in Materials and Methods, and *p53* mRNA relative expression level (REL). Statistical analysis was performed using the Mann–Whitney rank test.

Table 4. Relationship between mean gene expression (REL) and clinical stage

TNM staging*	Cases (42)	*c-raf* expression	*c-myc* expression	*c-jun* expression	*p53* expression
Tumour size					
T0	1	49.5	—†	0.23	—
T1	4	10.9	2.8	1.6	1.16
T2	12	23.4	3.1	1.5	0.84
T3	20	29.9	3.4	1.2	0.62
T4	5	16.2	3.5	1.7	0.41
Nodal status					
N0	19	26.3	3.3	1.2	0.84
N+	23	23.8	3.0	1.5	0.67
Metastasis					
M0	36	22.2	2.5	1.4	0.73
M1	6	37.5	5.6	1.3	0.65
Degree of differentiation					
Well	31	25.4	3.3	1.3	0.81
Moderate	5	24.2	4.9	1.6	0.64
Poorly	6	23.2	2.0	1.4	0.94

*According to UICC 1987; †not detected.

Table 5. Univariate analysis of tumour relapse after chemotherapy or radiotherapy

	Number of cases			
	Chemotherapy		Radiotherapy	
Criterion	Recurrence	Free of disease	Recurrence	Free of disease
c-myc REL*				
<2.4	8	3	5	6
$\geqslant 2.4$	7	6	2	1
P†	NS‡		NS	
c-jun REL				
<1.5	9	8	4	5
$\geqslant 1.5$	6	1	4	5
P	NS		NS	
c-raf REL				
<20	7	3	4	9
$\geqslant 20$	8	6	4	3
P	NS		NS	
p53 REL				
<0.6	12	5	3	3
$\geqslant 0.6$	3	4	6	4
P	NS		NS	
p53 status§				
Positive	4	6	2	3
Negative	11	3	4	4
P	0.05		NS	

*REL threshold as described in Results; †Statistical correlation between relapsed patients' group and non-relapsed patients' group with the population receiving either chemotherapy or radiotherapy; ‡NS, not significant; §Global status of *p53* with four antibodies.

c-jun), tumour suppressor gene *p53* and tumour size, nodal involvement, and presence of metastasis, histopathological features of the tumour (Tables 4 and 5), patient age and sex (data not shown).

There were statistically significant differences according to Mann–Whitney in tumour response to chemotherapy between patients with low or high *c-myc* expression ($P=0.0001$) and low or high *c-jun* expression ($P=0.0001$). Significantly

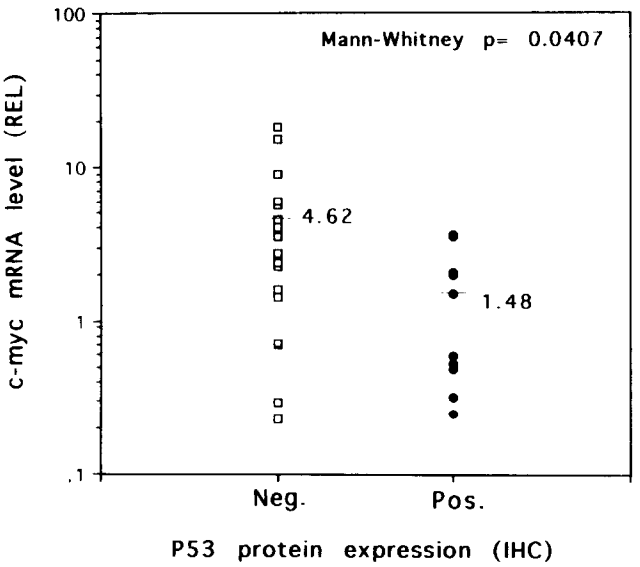

Fig. 2. Correlation between p53 protein expression measured by immunohistochemistry (IHC) noted as positive (pos.) or negative (neg.) as described in Materials and Methods, and *c-myc* mRNA relative expression level (REL). Statistical analysis was performed using the Mann–Whitney rank test.

decreased *c-myc* expression and increased *c-jun* expression were detected in patients' cells resistant to chemotherapy (Table 2). In addition, patients with complete response to radiotherapy displayed a lower level of *c-raf* oncogene compared to non-responding patients ($P=0.0494$ according to the Wilcoxon test) (Table 3). We determined oncogene expression (REL) thresholds in order to correlate these values with tumour relapse and disease-free survival. The thresholds (REL) were 2.4, 1.5, 20 and 0.6 for *c-myc*, *c-jun*, *c-raf* and *p53*, respectively.

There was no statistical correlation between p53 immunopositivity and response to chemotherapy and radiotherapy (χ^2 test $P=0.2$). However, lack of p53 protein expression was indicative of tumour relapse (χ^2 test $P=0.05$). In addition, there was a direct correlation between the absence of p53 protein expression and a high level of *c-myc* mRNA (Fig. 2) ($P=0.0407$ according to Mann–Whitney). There was no statistical difference in disease-free survival between all of these groups of patients. In spite of these results, we emphasise that we had relatively small numbers of patients and that the follow-up time is short.

DISCUSSION

Our results are derived from a prospective study undertaken to assess gene expression in patients with head and neck squamous cell carcinoma. Given the important role ascribed to oncogene and tumour suppressor gene abnormalities in the process of malignant transformation and resistance, we chose four probes to assess more accurately the frequency of overexpression in samples of fresh SCCHN tissue. These were chosen for the following reasons: *c-myc* RNA levels are associated with a reduced responsiveness to chemotherapy [15], *c-jun* is a component of the transcription factor AP-1, and its overexpression causes transformation without gene amplification [16], *c-raf* is associated with radioresistance in human laryngeal cancer [6] and *p53* is involved in the cellular response to DNA damage [17].

Overexpression of the *myc* gene occurs without amplification and has been reported by a number of authors [18]. However, in our study, *c-myc* expression is not correlated with prognostic variables, such as TNM stage or the number of involved lymph nodes. Interestingly, we found that decreased levels of *c-myc* RNA is associated with drug resistance of head and neck tumours. Since cells with high levels of c-myc protein are more prone to cell death upon serum deprivation, dysregulation of *c-myc* expression induces apoptosis in cells and growth arrest by a variety of means [6]. These finding are in disagreement with some reports in which *c-myc* gene overexpression at the mRNA and protein levels was linked to poor prognosis in SCCHN [19] as well as acquired drug resistance *in vitro* [20]. Using an enzyme-linked immunosorbent assay to quantitate the c-myc protein in SCCHN tumours, Field *et al.* were able to demonstrate statistically longer survival times in patients with lower levels of *c-myc* expression [19]. Other authors [4] have reported a definite correlation between low levels of c-myc protein in the tumour cells and metastatic lymph node involvement and advanced stages of SCCHN. To date, we can only postulate about the possible mechanism linking the low *c-myc* levels with aggressive tumour and chemoresistance behaviour. Our inverse correlation between lack of p53 protein expression and *c-myc* overexpression does not confirm the hypothesis that the growth suppression function of wild type *p53* can be bypassed by the activation of the *ras* and/or *myc* oncogenes [21] and that *p53* mutation should parallel *c-myc* overexpression, as shown in lung carcinoma [22].

The oncogene *c-jun* is part of the *jun* and *fos* families of oncoproteins which dimerise in the DNA-binding complex as components of the transcription factor, activation protein-1 (AP-1). The oncogene *c-jun* may be directly involved in tumorigenesis and there are reports of its expression in putative preneoplastic lesions induced by chemical carcinogens. These studies have reported significant expression of *jun* in tumours compared with a near absence of expression in normal liver [23]. In addition, it has been demonstrated that very low to undetectable levels of *c-jun* mRNA were found in normal tissue, like lung, brain, liver and thymus and that *c-jun* gene expression was induced by ethanol [24]. We report that *c-jun* mRNA was expressed in 100% of the SCCHN tumours, but this expression was not correlated to any biological parameters. However, this is the first report suggesting that tumours overexpressing *c-jun* are less susceptible to chemotherapy. Thus, it is known that *c-jun* is an immediate early response gene which is independently activated through stimulation, with a number of growth factors, during the G0–G1 phase of the cell cycle. It should be suggested that cells sustaining a high proliferation rate by growth factors and/or oncogenes could escape the apoptotic signal triggered by chemotherapeutic agents.

To date, numerous studies have explored changes in radiosensitivity caused by transfection with the *raf* oncogene [6] or related to the level of *raf* expression in tumour xenografts [25]. In our population, *c-raf* oncogene expression was not correlated to biological status of the tumour, drug-sensitivity or resistance, and disease-free survival. But we demonstrated that patients with low tumoral *c-raf* mRNA expression achieve complete response after radiotherapy ($P=0.0494$). This is in agreement with previous *in vitro* finding of Kasid *et al.*, who first identified the *raf* oncogene in a radiation-resistant cell line derived from a laryngeal squamous cell carcinoma [6].

Mutations of *p53* are one of the most common known

genetic defects in human cancer [26]. On the basis of sequence analysis of more than 300 tumours of various types studied so far, most appear to be missense and/or point mutations in conserved exons 5–9. The majority of *p53* mutations result in a conformationally altered but more stable protein which can be detected by immunohistochemistry. This approach has been used successfully to screen for *p53* mutations in breast, colorectal or lung cancer and also in SCCHN cell lines or tissues [8, 28]. Using four antibodies on Bouin fixed sections we identified a mutant immunophenotype in 42% of SCCHN tumours. In a large series of SCCHN we have previously reported the presence of a p53 protein alteration in 56% of primary tumour samples [14]. Using different molecular approaches, we have demonstrated that genetic alterations of *p53*, including abnormal mRNA expression, protein accumulation, occur frequently in SCCHN of all histological classes [29] which is similar to previous reports of Field *et al.* who found 63% of 73 cases of SCCHN overexpressing *p53* [30].

Quantitative or qualitative abnormalities in *p53* mRNA are relatively rare in malignant cells. Abnormally sized *p53* mRNA and the absence of *p53* mRNA expression have been observed in lung cancers [23]. However, we did not detect an abnormal transcript, as previously reported. Positive immunostaining has, in a number of reports, been correlated with factors associated with poor prognosis, early relapse or shortened disease-free survival in either monoparametric or multiparametric analysis. However, more recently, an opposite correlation was found with p53 immunostaining which predicts for favourable clinical prognosis [31]. In our population, there was a trend towards p53 positive immunostaining and reduced recurrence rate compared to p53 protein negative tumours, but this relationship was not statistically significant. This result confirms data reported by Volm *et al.* [32] suggesting that p53 immunostaining revealed by the monoclonal antibody PAb 1801, is correlated to a reduced recurrence rate in early-stage non-small cell lung cancer. Therefore, the prognostic impact of *p53* expression is probably linked to the effects of this protein on tumour cell proliferation and apoptosis.

The emerging picture of *c-myc*, *c-jun*, *c-raf* and *p53* gene function suggests that they may be involved in apoptosis inhibition in cancer treatment resistance. The concept of apoptosis inhibition is of great importance since: (a) tumour growth could be the result of a lengthening of the transformed cell survival by an apoptosis inhibitor rather than a stimulation of cellular proliferation; and (b) the consequence of programmed cell death inhibition is a resistance of the cancer cells to chemotherapy or radiotherapy. Although inference of the causal relationship in the context of apoptosis occuring or not occuring establishes the pivotal role of *p53*, *myc* and *Bcl2*, and some other genes, less clear-cut relationships concern genes whose expression coincides with programmed cell death, such as *raf* and/or *jun*. Since drugs and radiation cause apoptosis, this principle offers a new perspective on the therapeutic efficacy (or lack of it) of these treatments. It is known that expression of *Bcl2* and *p53* modulates drug and radiation toxicity. Therefore, we could define new important targets for anticancer drugs, such as genes known to be sufficient for malignant transformation and implicated in resistance (such as *p53*, *ras*, *raf* and *jun*) and genes whose expression is necessary, but not sufficient for malignant transformation (such as some cyclins). These rational approaches would allow a better targeted anticancer therapy [33].

1. Vokes EE, Weichselbaum RR, Lippman SM, Ki Hong W. Head and neck cancer. *New Engl J Med* 1993, **328**(3), 184–194.
2. Sakai E, Rikimaru K, Ueda M, *et al.* The *p53* tumor suppressor gene and *ras* oncogene mutations in oral squamous cell carcinoma. *Int J Cancer* 1992, **52**, 867–872.
3. Somers KD, Cartwright SL, Scheder GL. Amplification of the *ini-2* gene in human head and neck squamous cell carcinomas. *Oncogene* 1990, **5**, 915–920.
4. Gapany M, Pavelic ZP, Kelley DJ, *et al.* Immunohistochemical detection of c-myc protein in head and neck tumors. *Arch Otolaryngol Head Neck Surg* 1994, **120**, 255–259.
5. Evan GI, Wyllie AH, Gilbert CS, *et al.* Induction of apoptosis in fibroblasts by c-myc protein. *Cell* 1992, **69**, 119–136.
6. Kasid U, Pfeifer A, Weichselbaum RR, Dritschilo A, Mark GE. The *raf* oncogene is associated with radiation-resistant human laryngeal cancer. *Science* 1987, **237**, 1039–1041.
7. Burt RK, Garfield S, Johnson K, Thorgeisson SS. Transformation of rat liver epithelial cells with v-H-ras or v-raf causes expression of MDR-1, glutathione-S-transferase-P and increased resistance to cytotoxic chemicals. *Carcinogenesis* 1988, **9**(12), 2329–2332.
8. Yin XY, Smith ML, Whiteside TL, Johnson JT, Herberman RB, Locker J. Abnormalities in the *p53* gene in tumors and cell lines of human squamous cell carcinomas of the head and neck. *Int J Cancer* 1993, **54**, 322–327.
9. Lane DP. p53, guardian of the genome. *Nature* 1992, **358**, 15–16.
10. Sobin LH, Hermanek P, Hutter RV. TNM classification of malignant tumors. A comparison between the new (1987) and the old editions. *Cancer* 1988, **61**(11), 2310–2314.
11. Al Sarraf M. Head and neck cancer: chemotherapy concept. *Sem Oncol* 1988, **15**, 70–85.
12. Maniatis T, Fritsch EF, Aambrook J. *Molecular Cloning: a Laboratory Manual.* Cold Spring Harbor, Cold Spring Harbor Laboratory Press, 1982.
13. Tenaud C, Negoescu A, Labat-Moleur F, Legros Y, Soussi T, Brambilla E. p53 immunolabelling in archival paraffin-embedded tissues: optimal protocol based on microwave heating for 8 antibodies on lung carcinomas. *Modern Pathol* 1994, **7**(8), 853–859.
14. Lavieille JP, Brambilla E, Riva-Lavieille C, Reyt E, Charachon R, Brambilla C. Immunohistochemical detection of p53 protein in preneoplasic lesions and squamous cell carcinoma of the head and neck. *Acta Otolaryngol* 1995, **115**, 334–339.
15. Riou G. Proto-oncogene and prognosis in early carcinoma of the uterine cervix. *Cancer Surveys* 1988, **7**, 442–455.
16. Schütte J, Minna JD, Birrer MJ. Deregulated expression of human c-jun transforms primary rat embryo cells in cooperation with an activated *c-Ha-ras* gene and transforms Rat-1a cells as a single agent. *Proc Natl Acad Sci* 1989, **86**, 2257–2261.
17. Kastan MB, Onyekwere O, Sidransky D, *et al.* Participation of p53 protein in the cellular tesponse to DNA damage. *Cancer Res* 1991, **51**, 6304–6311.
18. Spandidos DM, Lamothe A, Field J. Multiple transcriptional activation of cellular oncogenes in human head and neck solid tumours. *Anticancer Res* 1985, **5**, 221–224.
19. Field JK, Spandidos DA, Stell PM, Vaughan ED, Evan Gi, Moore JP. Elevated expression of *c-myc* oncoprotein correlates with poor prognosis in head and neck sqamous cell carcinoma. *Oncogene* 1989, **4**, 1463–1468.
20. Delaporte C, Kragh Larsen A, Dautry F, Jacquemin-Sablon A. Influence of *c-myc* overexpression on the phenotypic properties of Chinese hamster lung cells resistant to antitumor agents. *Exp Cell Res* 1991, **197**, 176–182.
21. Oren M. p53. The ultimate tumor suppressor gene. *FASEB J* 1992, **6**, 3169–3176.
22. Gazzeri S, Brambilla E, Caron de Fromentel C, *et al.* p53 genetic abnormalities and c-myc activation in human lung carcinoma. *Int J Cancer* 1994, **58**, 24–32.
23. Sakai M, Okuda A, Hatayama I, *et al.* Structure and expression of the rat c-jun messenger RNA; tissue distribution and increase during chemical hepatocarcinogenesis. *Cancer Res* 1989, **49**, 5633–5637.
24. Kharbanda S, Nakamura T, Kufe D. Induction of the *c-jun* proto-oncogene by a protein kinase c-dependant mechanism during exposure of human epidermal keratinocytes to ethanol. *Biochem Pharmac* 1993, **45**(3), 675–681.

25. Rygaard K, Slebos RJ, Spang-Thomsen M. Radiosensitivity of small-cell lung cancer xenografts compared with activity of *c-myc*, *N-myc*, *L-myc*, *c-raf* and *K-ras* proto-oncogenes. *Int J Cancer* 1991, **49**, 279–284.
26. Hollstein M, Sidranski D, Vogelstein B, Harris C. p53 mutations in human cancers. *Science* 1991, **253**, 49–52.
27. Pavelic Z, Portugal L, Goote M, *et al.* Retrieval of p53 protein in paraffin-embedded head and neck tumor tissues. *Arch Otolaryngol Head Neck Surg* 1993, **119**, 1206–1209.
28. Matthews JB, Scully C, Jovanovic A, Van der Waal I, Yeudall WA, Prime SS. Relationship of tobacco/alcohol use to p53 expression in patients with lingual squamous cell carcinoma. *Oral Oncol, Eur J Cancer* 1993, **29B**, 285–289.
29. Riva-Lavieille C. Chimiorésistance, oncogènes et anti-oncogènes dans les tumeurs épithéliales. *Bull Cancer* 1994, **9**(suppl 2), 105s–111s.
30. Field JK, Pavelic ZP, Spandidos DA, Stambrook PJ, Jones AS, Gluckman JL. The role of the p53 tumor suppressor gene in squamous cell carcinoma of the head and neck. *Arch Otolaryngol Head Neck Surg* 1993, **119**, 1118–1122.
31. Passlick B, Izbicki JR, Riethmüller G, *et al.* p53 in non-small cell lung carcinoma. *J Natl Cancer Inst* 1994, **86**, 801–802.
32. Volm M, Mattern J. Immunohistochemical detection of p53 in non-small cell lung cancer. *J Natl Cancer Inst* 1994, **86**, 1249.
33. Gottesman MM. Report of a meeting: molecular basis of cancer therapy. *J Natl Cancer Inst* 1994, **86**, 1277–1285.

Acknowledgements—The authors thank Christine Claraz, Claude Chery and Laurent Azoti for performing the immunohistochemical staining and Christelle Deschaux-Blanc for statistical analysis. This work was supported by French Health Ministry, Ligue contre le Cancer, CAMPLP.