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Elevated expression of the c-*myc* oncoprotein correlates with poor prognosis in head and neck squamous cell carcinoma

J.K. Field[1], D.A. Spandidos[2, 3], P.M. Stell[4], E.D. Vaughan[5], G.I. Evan[6] & J.P. Moore[7]

[1] *Department of Clinical Dental Sciences, University of Liverpool, UK;* [2] *The Beatson Institute for Cancer Research, Garscube Estate, Bearsden, Glasgow, G61 1BD, Glasgow, Scotland, UK;* [3] *Biological Research Centre, The National Hellenic Research Foundation, 48 Vas-Constantinou Ave, Athens 11635, Greece;* [4] *Department of Otorhinolaryngology, University of Liverpool, UK;* [5] *Department of Maxillofacial Surgery, Walton Hospital, Merseyside, UK;* [6] *ICRF Laboratories, St Bartholomew's Hospital, Dominion House, 59 Bartholomew Close, London, UK;* [7] *Institute of Cancer Research, Chester Beatty Laboratories, Fulham Road, London, UK*

We quantitated c-*myc* oncoprotein in 44 squamous cell carcinomas of the head and neck using an enzyme-linked immunosorbence assay. The clinicopathological parameters of these patients were followed up for between 3 and 60 months and analysed for any correlations with observed levels of c-*myc* protein using the Kruskal-Wallis one-way analysis of variance method. Although no statistical correlation was found between different clinicopathological parameters (patient age, sex, TNM staging, number of lymph nodes invaded, extracapsular rupture of the tumour, its histopathological differentiation, or its site), the survival periods of patients with tumours possessing elevated levels of c-*myc* protein were found to be statistically shorter than those with lower levels of c-*myc* expression, ($P < 0.02$). This indicates that c-*myc* expression may be an effective prognostic indicator in head and neck cancer.

Introduction

Abnormal expression of oncogenes has been implicated in the development of cancer of the head and neck region. The involvement of the H-*ras*, K-*ras* and c-*myc* oncogenes in head and neck tumours has been analysed by Spandidos *et al.*, (1985) and by Field *et al.* (1986, 1987). Field *et al.* (1986) reported that elevated c-*myc* mRNA expression was correlated with advanced stages of the disease. Yokota *et al.* (1986) found c-*myc* amplification in two of seven squamous cell carcinomas (SCC) of the head and neck region during an extensive study of amplification in a wide range of tumours. They also found that the extent of c-*myc* amplification was relatively higher in all types of metastatic tumours (5–8 fold) than in primary tumours (3–5 fold). A correlation between c-*myc* amplification and extent of tumour metastasis was suggested.

The c-*myc* gene encodes a 62000 dalton nuclear protein, $p62^{c\text{-}myc}$, that appears to be involved in the control of cell proliferation, although its mechanism of action is uncertain (Bishop, 1987). Normal expression of the c-*myc* gene is tightly regulated, and this can occur at both transcriptional and post-transcriptional levels (Cole, 1986; Eisenman & Thompson, 1986; Alitalo *et al.*, 1987). Aberrant c-*myc* expression can arise from several causes, for example gene amplification, chromosomal rearrangement and, in some non-human systems, retroviral insertion (reviewed by Alitalo, 1987). Amplification of the c-*myc* gene has been reported in many tumours including small cell carcinoma of the lung (Little *et al.*, 1983; Johnson *et al.*, 1988); mammary carcinoma (Kozbor & Croce, 1984; Escot *et al.*, 1986; Varley *et al.*, 1987); in a variety of other carcinomas (Alitalo *et al.*, 1983; Yokota *et al.*, 1986); and in human leukaemia (Collins & Groudine, 1982; Dalla-Favera, 1982), and it is associated with high levels of c-*myc* expression. Elevated levels of c-*myc* mRNA, relative to that seen in normal fibroblasts, have also been reported in a range of solid tumours derived from the kidney, renal, breast, lung, colon, caecum, small bowel, ovary and uterine cervix carcinoma tissues (Slamon *et al.*, 1984; Kozbor & Croce, 1984; Varley *et al.*, 1987; Riou *et al.*, 1987; Escot *et al.*, 1988; Erisman *et al.*, 1988a), often in the absence of amplification of the gene. Thus, elevated c-*myc* mRNA levels, irrespective of the mechanisms by which they occur, are associated with diverse neoplastic disease.

Most reports quantitating c-*myc* expression in tumours have relied on measurement of c-*myc* mRNA. However, such analyses yield no direct information on the levels of the c-*myc* protein in the tissue and, moreover, are complicated by RNA degradation *post mortem* in archival tumours. Methods used previously to assay the c-*myc* oncoprotein in tumour samples have been time consuming and generally insensitive and non-quantitative (e.g. Sikora *et al.*, 1985; Stewart *et al.*, 1986). Moore *et al.*, (1987) have recently developed an ELISA for $p62^{c\text{-}myc}$ which is rapid, reproducible, sensitive and quantitative. This assay has been used to measure the levels of c-*myc* protein in normal and transformed cell lines (Moore *et al.*, 1987; Erisman *et al.*, 1988b).

The aim of this investigation was to determine by ELISA whether differential amounts of c-*myc* oncoprotein can be measured in squamous cell carcinoma from the head and neck, and hence to assess whether levels of c-*myc* oncoprotein in the tumours correlate with clinicopathological data.

Results

C-myc *expression in xenografts containing Colo 320 HSR cells*

The cell line Colo 320 HSR, a human colonic apudoma, has a 30 fold amplification of the c-*myc* gene (Alitalo *et*

Correspondence: J.K. Field, Department of Clinical Dental Sciences, University of Liverpool, PO Box 147 Liverpool, UK.

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Table 1a Elevated levels of c-*myc* oncoprotein in patients with SCC of the head and neck

Patient number	*pg c-myc/μg tp tumour*	*Site of tumour*	*TNM*	*Tumour type*	*Histopathology grade*	*pN*	*ECR*	*Fate*
5	13.25	2	40	P	3	0	0	7R, 10DOD
7	9.00	3	33	P	1	8	0	3R, 9DOD
1	8.84	1	30	R	2	0	0	8DOD
25	2.73	2	43	P	2	NA	NA	4DOD
43	2.5	3	32	P	2	1	0	9DOD
16	2.3	1	22	R	1	5	0	4R
6	2.0	1	41	P	2	0	0	11
3	2.0	3	30	P	1	0	0	13
21	1.96	4	02	R	3	2	0	8
15	1.9	2	22	P	1	NA	NA	3R
17	1.84	3	40	R	2	3	1	4DOD
24	1.8	2	30	P	1	2	1	8DOD
23	1.6	1	22	P	2	3	3	7
20	1.51	4	02	R	2	NA	NA	8
30	1.14	1	10	P	2	0	0	6
2	1.13	1	21	R	1	NA	NA	3D
22	1.06	1	10	P	2	0	0	7
26	1.04	2	40	P	2	0	0	3
44	1.0	1	33	P	2	4	0	26DOD
34	0.99	3	20	P	1	0	0	7
14	0.98	1	20	R	1	0	0	57

Table 1b Patients without elevated c-*myc* expression in SCC of the head and neck

Patient number	*pg c-myc/μg tp tumour*	*Site of tumour*	*TNM*	*Tumour type*	*Histopathology grade*	*pN*	*ECR*	*Fate*
4	0.79	2	11	R	2	3	0	11
42	0.75	4	30	R	3	0	0	8
31	0.70	1	42	P	1	0	0	5
41	0.65	1	42	P	2	3	1	6
29	0.49	1	20	R	1	NA	NA	7
9	0.48	4	03	R	2	1	0	15
28	0.44	2	20	P	2	0	0	4
27	0.41	3	01	R	2	7	1	3
10	0.38	4	03	R	3	4	1	1DOD
13	0.32	3	30	R	1	0	0	57
35	0.27	4	20	R	3	0	0	8
8	0.26	4	01	R	1	2	1	10
12	0.25	3	30	P	2	0	0	60
33	0.22	1	10	P	1	1	0	7
36	0.21	2	33	R	2	1	0	3
32	0.21	1	42	P	2	0	0	5
19	0.18	4	02	R	2	5	2	6R
38	0.17	4	03	R	2	0	0	8
40	0.14	1	22	P	2	0	0	7
18	0.14	1	21	P	2	0	0	9
39	0.12	4	01	R	2	0	0	7
37	0.12	3	22	P	2	1	1	3R
11	0.11	2	21	R	3	5	0	1D

Site of tumour: 1 = Oral cavity 2 = Pharynx 3 = Larynx 4 = Node
Tumour type: P = Primary R = Recurrence
Histopathological grade: 1 = well differentiated SCC; 2 = moderately differentiated SCC; 3 = poorly differentiated SCC
pN: Lymph node metastasis at pathology
ECR: Encapsular rupture
Fate: Number of months between date of operation at which tissue was obtained for c-*myc* analysis and the date of follow-up
DOD = died of disease
R = recurrence
D = died of other causes
NA: Data not available

Elevated expression of the c-*myc* oncogene is 0.91 pg c-*myc*/μg tp. (2 standard deviations above the mean of 22 specimens of normal tissue)

al., 1983) and expresses c-*myc* protein at high levels (Evan *et al.*, 1985). Xenografts of Colo 320 HSR cells, grown in nude mice, were used as a positive control in these studies. The median c-*myc* oncoprotein level in 9 extracts of a murine xenograft containing Colo 320 HSR cells was 14.55 (range 6.8–21.88) pg c-*myc* protein/μg total protein (tp), whereas normal tissue (stratified squamous epithelium) from a nude mouse had a median value of 0.26 (range 0.24–0.28) pg of c-*myc* protein/μg tp. This indicated a 55-fold increase in c-*myc* expression in the xenograft tissue compared with normal tissue, consistent with a 30-fold amplification of the c-*myc* gene found in Colo 320 HSR cells. A 40-fold elevation in c-*myc* mRNA and a 54-fold increase in c-*myc* protein in Colo 320 HSR cells in tissue culture have also been reported (Moore *et al.*, 1987; Erisman *et al.*, 1988b). These experiments therefore indicate that the extraction procedure is valid for estimating the amount of $p62^{c\text{-}myc}$ in solid tumours.

C-myc *expression in normal tissue*

(*i*) *From non-cancer patients* Normal tissue (stratified squamous epithelium) was obtained from four patients with no known cancer. These specimens were assayed for c-*myc* oncoprotein expression using the ELISA technique and were found to have a median value of 0.22 (range 0.18–0.25) pg c-*myc* protein/μg tp.

(*ii*) *Normal tissue taken from the resection margin in head and neck cancer patients* Tissue specimens were taken from the resection margin in 18 patients undergoing head and neck surgery and have been described as histologically normal. These specimens were assayed for c-*myc* oncoprotein using the ELISA technique and found to have a median value of 0.37 (range 0.2–1.1) pg c-*myc* protein/μg tp.

C-myc *expression in head and neck SCC tissue lysates*

Tissue lysates for $p62^{c\text{-}myc}$ determination were prepared from 44 samples of squamous cell carcinoma. The details of these patients are given in Table 1. Specimens were taken from 23 patients who had a previously untreated SSC, and 21 specimens were taken from patients who had a recurrence at the time of surgery. The median value of c-*myc* oncoprotein expression in the 23 previously untreated SCC was 1.04 pg c-*myc* protein/μg tp (range 0.12–13.25) and the median value for the patients with recurrences was 0.48 pg c-*myc* protein/μg tp (range 0.12–8.84). These data were tested by the Kruskal-Wallis one-way analysis of variance method (Siegel, 1956) and it was found that there was no significant difference in $p62^{c\text{-}myc}$ expression between these two groups of patients. For this reason we felt it appropriate to group together the patients with previously untreated tumours and those with recurrences in the following analyses.

The clinicopathological parameters detailed in Tables 1a and 1b were compared with c-*myc* expression using the Kruskal-Wallis one way analysis of variance. The level of $p62^{c\text{-}myc}$ in the tissue (Table 2) was not statistically correlated with patients age, sex, TNM staging (UICC, 1978), pathological lymph node metastasis (pN) or the degree of the tumour's extracapsular rupture (ECR), lymph node metastasis, histopathological differentiation, or site.

In this report we consider the c-*myc* oncogene to be overexpressed if the $p62^{c\text{-}myc}$ level is two standard deviations above the mean value of 22 specimens of normal tissue (i.e., 4 normal oral specimens from patients with no evidence of disease and 18 specimens of normal tissue taken from the resection margin in head and neck operations). This mean value is 0.38 ± 0.27 pg c-*myc*/μg tp (mean $\pm$ SD), and so overexpression is defined as more than 0.91 pg c-*myc* protein/μg tp, [0.38 + (1.96 × 0.27)] (Table 3).

Table 2 Correlation of c-*myc* expression in SCC of the head and neck

	c-myc *expression*				
	n	*Median*	*Range*	χ^2	*df*
T Status					
1	4	0.92	(0.22–1.14)	7.16	4
2	13	0.49	(0.11–2.30)		
3	10	1.40	(0.21–9.00)		
4	8	1.44	(0.21–13.25)		
	9	0.38	(0.12–1.96)		$P > 0.05$
N Status					
0	17	0.99	(0.22–13.25)	3.03	3
1	8	0.33	(0.22–2.00)		
2	12	1.10	(0.12–2.50)		
3	7	0.48	(0.17–9.00)		$P > 0.05$
Type					
Primary	23	1.05	(0.12–13.25)	1.61	3
Recurrent	21	0.44	(0.11–8.84)		$P > 0.05$
Histology					
Well	13	0.99	(0.22–9.00)	1.59	2
Moderate	25	0.65	(0.12–8.84)		
Poor	6	0.56	(0.11–13.25)		$P > 0.05$
Site					
1	16	0.99	(0.14–8.84)		
2	9	0.79	(0.11–13.25)		
3	9	0.99	(0.17–9.00)	1.6	3
N	10	0.37	(0.12–1.96)		$P > 0.05$

T and N status —(UICC, 1978)
Type of tumour—Primary tumour; Recurrent tumour.
Histology —Well differentiated: Moderately differentiated: Poorly differentiated: Squamous cell carcinoma.
Site —Site of tumour: 1 = Oral: 2 = Pharynx: 3 = Larynx: N = nodal.

Table 3 Median and range of c-*myc* expression in patients with SCC

	Elevated c-myc *protein level* (*>0.91 pg* c-myc *protein/μg tp*)*			c-myc *protein not elevated* (*< 0.91 pg* c-myc *protein/μg tp*)		
	No.	*Median*	*Range*	*No.*	*Median*	*Range*
Total No. patients	21	1.84	(0.98–13.25)	23	0.26	(0.11–0.79)
Died of the disease	8	2.6	(1.00–13.25)	1	0.38	
Died of other causes	1	1.13		1	0.11	

* See Table 1 for definition of elevated level of c-*myc* protein

Table 4 Survival analysis between patients with elevated and non-elevated levels of c-*myc* expression

	χ^2	*df*	*P*
(a) Calculated from date of the operation when tissue was obtained for c-*myc* analysis	5.85	1	<0.02
(b) Calculated from date the disease was first diagnosed	7.92	1	<0.01

See Table 1 for definition of elevated c-*myc* expression

Survival data

Survival calculations were based on the time interval between the date of the operation at which tissue was obtained for c-*myc* analysis and the date of follow up, irrespective of whether the patient initially had a previously untreated or a recurring tumour at the time of the operation. The 44 SCC patients were separated into two groups: those with tumour c-*myc* levels higher than, and those with tumour c-*myc* levels lower than, 0.91 pg c-*myc* protein/μg tp. The survival analysis (Peto *et al.*, 1976) was statistically different for patients with low levels and over-expressed levels of c-*myc* ($\chi^2 = 5.85$; $P < 0.02$) (Table 4).

Since the clinical and biological reasons for recurrences are uncertain, survival was also calculated from the time of first diagnosis of the SCC. This period was again found to be statistically different in the two groups ($\chi^2 = 7.92$; $P < 0.01$), (Peto *et al.*, 1976) (Table 4). Survival curves calculated from the time of first diagnosis of SCC are shown in Figure 1.

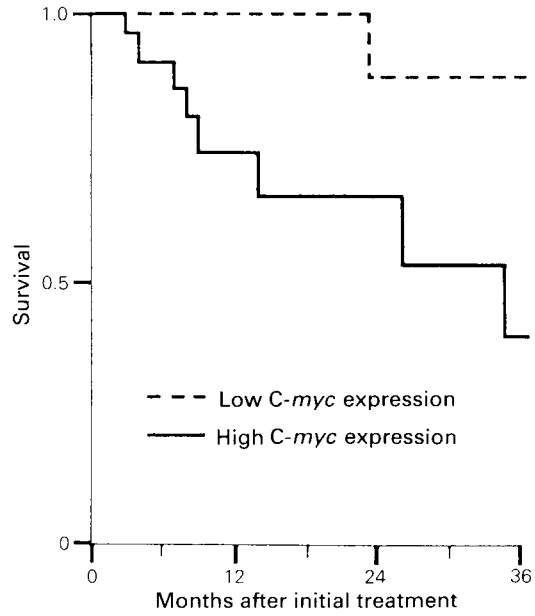

Figure 1 Survival curves are drawn up using the Kaplan-Meier product limit estimates (Kaplan & Meier, 1958). This survival curve is calculated from the date the disease was first diagnosed

Discussion

In this study we have shown that high level expression of the c-*myc* protein in tumour cells from patients with SCC of the head and neck correlates with poor prognosis.

To date, a number of prognostic indicators has been used to predict the clinical course of squamous cell carcinoma of the head and neck. These are grouped under three headings: (1) Patient parameters; (2) Tumour parameters; and (3) Treatment parameters (reviewed by Davis, 1985). All of these are interrelated in determining the patient's prognosis and attempts have been made to separate out the most important components.

Patient parameters may be divided into age, sex, race, nutritional and immunological status. The most useful grouping of these variables has been the SALPI system, (stage, age, lymphocyte prognostic indicator), which has been found to have an overall rate of successful prediction of 80% (Katz, 1983).

Major parameters pertaining to the tumour are the site and size of the primary tumour and the presence or absence of regional or nodal metastasis. The Union Internationale Contre le Cancer (UICC, 1978) and the American Joint Committee on Cancer Staging (AJC, 1978) have used these parameters to describe the TNM system, which is probably the best current prognostic indicator, especially for stage I and IV patients. The system is less efficacious with stage II and III patients. In addition to the TNM system, *individual* tumour parameters such as lymph node status, histological differentiation of the tumour and histology of lymph nodes have each been investigated for prognostic usefulness. In cancer of the oral cavity, the presence of a single histologically-positive lymph node metastasis is the most important factor in determining prognosis (Kalnins *et al.*, 1977) as it decreases the 5-year expectation by 45%. Histological grading systems and nuclear parameters have also been reported (Davis, 1985). DNA content was measured by cytophotometric analysis of Feulgen-DNA from tumour specimens, and classified into diploid or non-diploid. It was found that patients with diploid or low DNA values had a better prognosis than those tumours of non-diploid or with high DNA values (Holm *et al.*, 1982). It is of interest that DNA analysis of tumour was more specific than histology with respect to low and high clinical stages, and also between carcinomas that metastasise and those that do not.

More recently, oncogene expression has been considered as a prognostic indicator in a variety of tumours. Specifically, amplification and over-expression of the various *myc* genes has been reported to be prognostic indicators in certain tumours. Amplification of the N-*myc* gene correlates with degree of aggression of the neuroblastoma (Brodeur *et al.*, 1984) and the progression of this disease is also associated with amplification and over-expression of N-*myc* (Schwab *et al.*, 1984; Brodeur *et al.*, 1987). C-*myc* amplification in patients with breast cancer has been shown to indicate a very poor short term prognosis (Varley *et al.*, 1987).

To gain a further understanding of the importance of the c-*myc* gene in the progression of the disease, we have analysed the expression of the c-*myc* oncogene product by ELISA in head and neck cancer. The 44 SCC specimens were made up from 23 previously untreated tumour samples and 21 recurrences or metastases. There is no significant difference between the levels of c-*myc* expression in these two groups, and we find no correlation between levels of c-*myc* protein and the patient age, sex, TNM staging, site of tumour, histopathological grading, lymph node metastasis or encapsular rupture.

The levels of c-*myc* oncoprotein expression in the 22 specimens of normal tissue analysed in this study are found to be normally distributed, whereas levels in the 44 SCC specimens are not normally distributed, being right-skewed. Over-expression of c-*myc* is therefore defined in this report as two standard deviations above the mean value found in the normal tissue specimens (i.e. >0.91 pg c-*myc*/μg tp).

The survival period of the patients with c-*myc* over-expression is significantly less than those without elevated expression (Table 4). We have calculated the survival times on the basis that the c-*myc* gene was over-expressed at the time of the surgery (regardless of whether it was a primary or a recurrent tumour), and we have also calculated from the time of diagnosis in the case of recurrent tumours. The latter calculation was undertaken to investigate whether there would be a significant difference in the survival data if it were assumed that the c-*myc* gene was over-expressed from the time of diagnosis. No matter which method is used to calculate the survival times, the inverse correlation between the level of c-*myc* expression and survival still holds. In this investigation we demonstrate that c-*myc* protein levels in a tumour appear to be a very good prognostic indicator. Only the clinical 'N' status, gives comparable survival curves (Stell, 1989). In this study we also find no correlation between any of the established prognostic factors (i.e. TNM, histology etc.) and c-*myc* expression, and it is therefore proposed that c-*myc* expression may be an independent prognostic indicator in head and neck cancer.

The molecular mechanisms causing elevated levels of the c-*myc* protein in more aggressive SCC of the head and neck are unknown. We do not know whether the over-expression is a cause or consequence of carcinogenesis. Nonetheless, the level of c-*myc* protein does appear to be a strong prognostic indicator, even within the somewhat limited follow up period for some of these patients. These data indicate that clinical trials incorporating measurements of c-*myc* protein expression may be of value. Prospective studies will also be necessary to establish the importance of these observations and we are presently in the process of setting them up.

Materials and methods

Surgical tissue specimens

Forty eight specimens were taken at the resection margin from patients undergoing head and neck surgery. Tissue specimens were snap-frozen within 20 minutes of surgical removal, and histological analysis was undertaken on the tumour specimens. It was possible to take normal tissue specimens from 18 patients undergoing head and neck surgery. This normal tissue was taken at least 2 cm from the tumour, at the resection margin, and was reported histologically normal. In addition, four normal tissue specimens were taken from the oral region of patients with no known cancer. The clinicopathological parameters for all patients were available; age, sex, TNM staging (UICC, 1978), pathological lymph node metastasis, extracapsular rupture of the lymph node metastasis, histopathological differentiation, site of tumour and follow up (3–60 months).

Xenograft tissue specimens

A xenograft of Colo 320 HSR cells was prepared in a Balb/c nude mouse and used to test the efficiency of the technique used to extract c-*myc* protein from solid tumours.

Tissue lysate preparation

The preparation of tissue lysates for $p62^{c\text{-}myc}$ determination was performed as described by Moore *et al.* (1987), with minor modifications. Finely minced tissue was lyophilyzed and resuspended in lysis buffer (144 mM NaCl, 25 mM Tris HCl pH 7.5 (TBS) containing 1% SDS, 1% aprotinin, 0.5 mM phenyl methyl sulpharyl fluoride [PMSF] and 50 mM dithiothreitol [DTT] at a concentration of 10–20 mg tissue (wet weight) per 100 μl. The suspension was boiled for 5 min, incubated for 30 min on ice with 100 mM iodoacetamide, then diluted with 9 volumes of TBS/NP40 (TBS containing 1% Nonidet P40). Insoluble material was recovered by centrifugation (1 min, 14 000*g*). The protein concentration in the extract was determined by the method of Bradford (1976) using a BioRad protein kit. Three specimens of each normal and tumour samples were analysed for c-*myc* oncoprotein expression (unless there was a shortage of tissue) and the mean values in pg c-*myc*/μg total protein are given in Table 1.

C-myc *oncoprotein measured by ELISA*

The method has been described in detail elsewhere (Moore *et al.*, 1987) but in summary it involves the following steps. Pan-*myc* antibody (Evan *et al.*, 1988) adsorbed to microtitre wells captures c-*myc* protein from a tissue lysate prepared as described above. Captured protein is then recognised by a second anti-c-*myc* antibody MYC1-3C7 (Evan *et al.*, 1985) conjugated to alkaline phosphatase. The amount of bound alkaline phosphatase, proportional to the amount of c-*myc* protein in the extract, is detected colorimetrically using an extremely sensitive enzymatic system (AMPAK, Novo BioLabs, Cambridge, UK) which involves the following: in the first step, alkaline phosphatase dephosphorylates NADPH to NADH. In the second or amplification step, a pair of cycling redox reactions utilises NADH to reduce a colourless precursor to a coloured formazan dye (Self, 1985; Stanley *et al.*, 1985; Johannsson *et al.*, 1986). The reaction is stopped with acid and the optical density determined at 492 nm using a standard plate reader. The assay is calibrated with reference to known amounts of bacterially expressed c-*myc* protein (Watt *et al.*, 1985).

Statistical analysis

The values for c-*myc* oncoprotein expression of the 44 squamous cell carcinomas are not normally distributed, being right skewed. Non-parametric methods were used throughout and data for c-*myc* oncoprotein expression are therefore displayed as median and range. Differences between groups were analysed by the Kruska-Wallis one-way analysis of variance for non-parametric data (Siegel, 1956). Survival curves are drawn up using the Kaplan-Meier product limit estimate (Kaplan & Meier, 1958). Differences between survival times were analysed by the log rank method (Peto *et al.*, 1976).

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