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Papers

Stage of Disease Confounds Apparent Relationship Between Levels of N-ras and Duration of Survival in Head and Neck Tumours*

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The objective of this study was to determine whether elevated levels of N-ras correlated with clinicopathological data. Complete clinical data were available on 133 of 481 patients surgically treated for squamous cell carcinoma of the head and neck (SCCHN) who had immunohistochemical data for N-ras. Advanced stages of disease were strongly related to the staining for N-ras in tumour cells ($P=0.0031$). The stage of disease was inversely related to duration of survival ($P=0.0017$). Initial statistical evaluation revealed an apparent correlation between survival and N-ras staining. However, duration was found to be *independent* of the level of N-ras. The illusory relationship initially was a result of the confounding effect of the stage of disease. Copyright © 1996 Published by Elsevier Science Ltd

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INTRODUCTION

Prognostication of squamous cell carcinoma of the head and neck (SCCHN) utilising the TNM classification is unreliable. Investigation into other clinicopathological variables, such as DNA ploidy, histological grade, nutritional and immunological status, sex, and age have similarly failed to predict outcome in terms of survival [1–3].

Recently, attention had been focused on cellular oncogenes as potential prognostic predictors of malignant tumours [4–8]. The *ras* family of cellular oncogenes (*H-ras*, *K-ras*, *N-ras*) are among the most frequently detected transformation-inducing genes in human solid tumours [9, 10]. The RAS protein p21, a 21 000 Da protein located on the internal surface of the cytoplasmic membrane, binds GTP (active state) and GDP (inactive state) and possesses GTPase activity. Ras proteins also participate in signal transmission (via RASGAP proteins and neurofibromin), activate the RAF-MEK-ERK kinase cascade, and bind phosphatidylinositol-3-OH kinase (growth control enzyme) when in the active state [4–12]. While they are clearly implicated in the control of growth and development of cells, the full extent of ras functions remains unknown [9]. It has been reported that the *ras* genes can be activated by point mutations occurring at codons 12, 13, or 61, resulting in decreased GTP hydrolysis and an increase in oncogenic potential [10].

In a recent study from this department, McDonald *et al.* [13] showed that the overexpression of the *ras* family of oncogenes may play a role in late stages of tumorigenesis. The purpose of this retrospective study is to determine whether elevated levels of N-ras, detected by immunohistochemistry, correlate with clinicopathological data.

MATERIALS AND METHODS

Tissue specimens

We reviewed an archival collection of 481 SCCHN specimens compiled from the University of Cincinnati Medical Center and the Cincinnati Veterans Administration, Cincinnati, Ohio, U.S.A., However, complete clinical data were only available on 133 patients surgically treated between 1980 and 1992. The post-treatment survival time ranged from 1 to 153 months. All patients had only a single primary tumour and did not receive prior treatment. There were 89 males and 44 females ranging in age from 34 to 88 years. The patient distribution by primary site of the tumour was as follows: oral cavity, 84; larynx, 31; hypopharynx, 10; oropharynx, 6; sinonasal, 2.

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Reagents

Mouse monoclonal antibodies against N-ras (clone F155-277) were purified from ascites fluid by non-denaturing liquid chromatography and supplied by Oncogene Science, Inc. (Uniondale, New York, U.S.A.). The immunogens used to generate these antibodies were bacterially derived recombinant fusion proteins constituting residues 66–189 of the respective human c-RAS p21s and a portion of the trpE gene product. The specificities of the N-ras antibodies have been confirmed and reported by Paleywala and Goldsmith [14]. These antibodies react by either immunoprecipitation, immunoblotting or in histopathology procedures with human and other mammalian C- and V-p21, but not with each other nor with mutated ras protein. These antibodies were used at 1:20 dilution (final concentration 5 μg). Negative controls were tissues incubated with the same dilution of mouse IgG1. Other reagents used were rabbit anti-mouse immunoglobulins (Dako Corp., Carpinteria, California, U.S.A.); peroxidase–anti-peroxidase (PAP) mouse monoclonal complex (Dako Corp.); and normal human serum (Gibco BRL, Grand Island, New York, U.S.A.).

Immunohistochemistry

The retrieval technique previously described by Shi *et al.* [15] and proved effective in head and neck carcinoma by Pavelic *et al.* [16], was utilised prior to performing immunohistochemical analysis. Endogenous peroxidase activity in tissue samples was neutralised by 15 min incubation in 3% H_2O_2 in methanol. The slides were washed in phosphate-buffered saline (PBS) and non-specific binding was blocked by applying normal rabbit serum in a humidity chamber at a dilution of 1:10 for 30 min. The slides were blotted and non-specific binding was blocked by applying normal rabbit serum in a humidity chamber at a dilution of 1:10 for 30 min. The slides were blotted and the primary anti-ras antibodies in the appropriate optimised solution were applied for 2 h at room temperature. The slides were then washed three times in PBS containing 3, 2, and 1% normal human serum (NHS), respectively. The second antibody (rabbit anti-mouse immunoglobulin) diluted 1:25 with PBS containing 1% bovine serum albumin (BSA) and 1% NHS was applied for 1 h at room temperature. Finally, PAP-conjugate diluted 1:100 in PBS with 1% BSA was applied for 1 h. The slides were washed in PBS, and incubated for 10 min in diaminobenzidine tetrahydrochloride used as a chromagen. Prior to mounting, the slides were counterstained with haematoxylin.

Evaluation of slides

Tissue sections were evaluated randomly in a double blind fashion for the presence or absence of N-ras protein using light microscopy. Two experienced pathologists concurred on the evaluation of each specimen. A section was considered positive if greater than 1% of cells exceeded background levels of staining.

RESULTS

Figure 1 shows that the stage of disease was the most powerful predictor of survival ($P=0.0017$). Age was the next greatest predictor of survival duration (data not shown). Tumour size, metastasis (to regional lymph nodes), and gender were not significantly related to months of survival (data not shown).

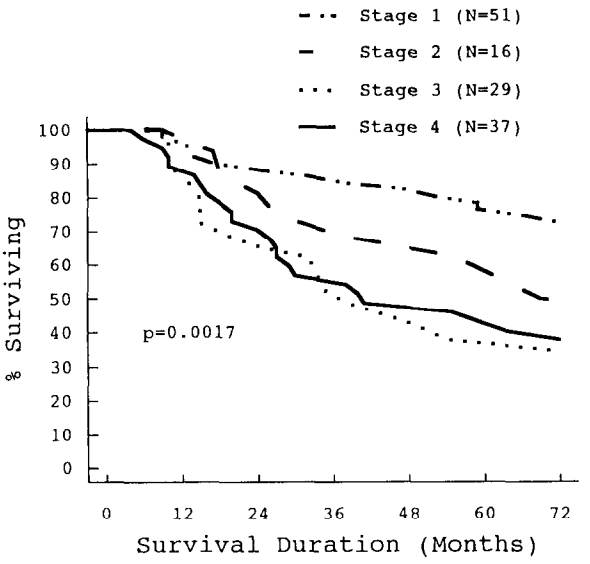

Fig. 1. Note the highly significant relationship between survival and clinical stage of disease ($P=0.0017$).

Table 1. Relationship between stage of disease and immunohistochemical staining for N-ras protein (chi-square $P=0.0031$)

	Stage of disease				
	1	2	3	4	Totals
N-ras Absent	36	7	11	13	67 50.1%
N-ras Present	15	9	18	24	66 49.9%
Total	51 38.4%	16 12.0%	29 21.8%	37 27.8%	133 100%

The relationship between an increased stage of disease and elevated levels of N-ras was also highly significant ($P=0.0031$), as indicated by chi-square analysis (Table 1). Additionally, subdividing tumour tissues positively stained for N-ras protein into groups of low or high intensity staining, resulted in a significant positive correlation between intensity. Increased expression of N-ras significantly correlated with duration of survival upon initial analysis. However, this result was due to the confounding effect of disease stage, which is strongly related to both the expression of N-ras and the duration of survival. Survival is not significantly related to N-ras levels when population is stratified by stage of disease (Fig. 2).

DISCUSSION

In this report we corroborate the finding that the presence of N-ras protein in SCCHN appears to be involved in the late stages of the malignant process. Saranath *et al.* [17] reported on 23 patients in which 3 were found to be N-ras positive with stage III disease and 4 with stage IV disease. None were positive with stage I or II disease. Similarly, McDonald *et al.*

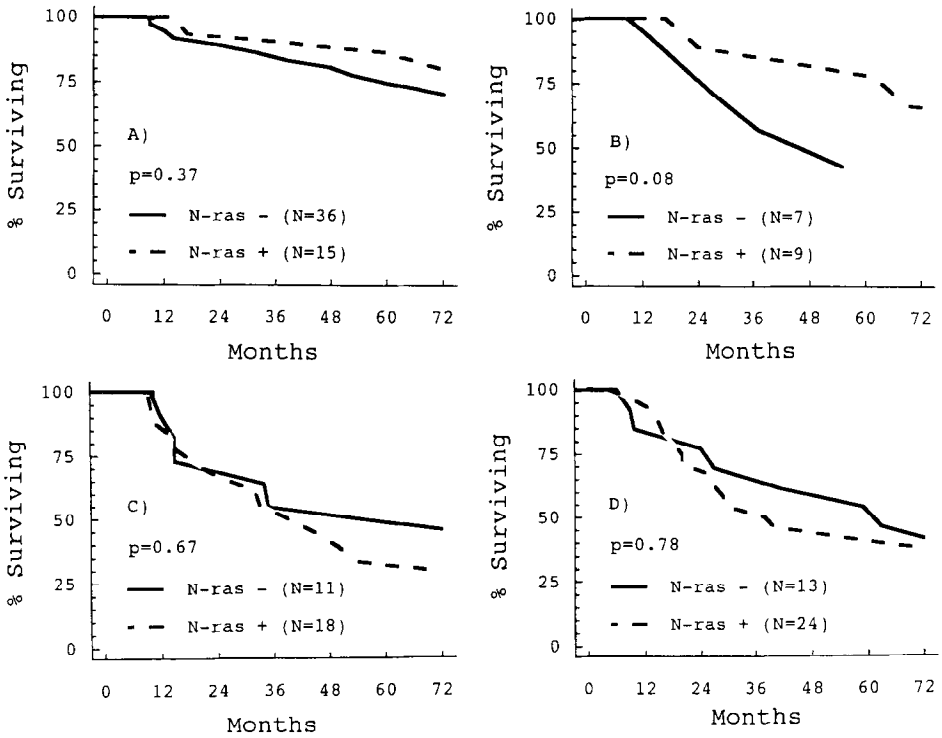

Fig. 2. Survival is not significantly related to N-ras staining when the population is stratified by stage of disease. (A = Stage I; B = Stage II; C = Stage III; D = Stage IV).

[13] reported on 22 patients with squamous cell carcinoma of the oral cavity and found an association with N-ras and late stage disease (stage III, IV). We conclude, based on this and other studies, that N-ras alteration is a late stage event in head and neck carcinogenesis.

Upon initial review of these data, it was determined that increased levels of N-ras protein correlated significantly ($P < 0.05$) with decreased duration of survival. However, upon further review, it was concluded that increased stage of disease, not expression of N-ras, correlated with survival ($P = 0.0017$). This confounding effect is of tremendous clinical significance. Erroneous deductions regarding clinical correlation between survival and the presence of a proto-oncogene protein can occur without notice. Careful statistical analysis must be undertaken before such conclusions are stated. Tumour size (T stage), regional metastasis, age and gender were also evaluated for impact on survival. Age proved to be the only clinical factor that impacted on survival ($P = 0.01$).

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