Pergamon

Oral Oncol, Eur J Cancer, Vol. 31B, No. 1, pp. 63–67, 1995

0964–1955/95 $9.50 + 0.00

0964–1955(94)00034–4

Heat Shock Protein Expression in Oral Epithelial Dysplasia and Squamous Cell Carcinoma

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Heat shock protein (HSP) expression is upregulated in tumour cells and, therefore, HSP expression is a likely marker of the malignant potential of oral epithelial lesions. Furthermore, the 70-kDa HSP (HSP 70) is implicated in the degree of tumour differentiation, the rate of tumour proliferation and the magnitude of the anti-tumour immune response. Accordingly, the distribution and intensity of HSP 70 expression was assessed in the epithelial compartment of oral squamous cell carcinoma (SCC, $n=29$), dysplastic oral epithelium ($n=18$) and benign oral mucosal lesions ($n=22$) using avidin–biotin complex immunohistochemistry and microdensitometry under standardised conditions. Staining intensity was recorded in kilo-ohms (kΩ). Normal oral mucosa ($n=15$) was used for comparison, and results were analysed using Kruskall–Wallis and Fisher's exact tests. The distribution of HSP 70 expression in well differentiated SCC was significantly different from that in poorly differentiated SCC ($P<0.05$), the latter demonstrating a more focal staining pattern. Median staining intensity in SCC (6.22 kΩ), epithelial dysplasia (9.61 kΩ) and the benign oral mucosal lesions (8.28 kΩ) was significantly greater than that in normal oral mucosa (5.64 kΩ; $P<0.05$). Staining intensity in poorly differentiated SCC (7.66 kΩ) was greater than that in moderately differentiated SCC (4.77 kΩ), although this result just failed to reach statistical significance ($P=0.06$). These results suggest that, as employed currently, HSP 70 expression is not a definitive marker of oral malignancy or malignant potential. However, with further development, quantitative analysis of anti-HSP 70 immunoreactivity may be valuable as an adjunct to conventional histology for assessing the malignant potential of oral mucosal lesions.

Keywords: oral mucosal disease, epithelial dysplasia, squamous cell carcinoma, heat shock protein

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INTRODUCTION

OF FUNDAMENTAL concern in oral pathology is the ability to distinguish dysplastic epithelial lesions that will progress to malignancy from those that will not [1]. At present, there is no definitive, quantifiable marker allowing this distinction although it is generally considered that lesions with more severe dysplasia have greater malignant potential, and that poorly differentiated malignancies have a worse prognosis.

Previous studies have shown that tumour cells, *in vivo* and *in vitro*, express elevated levels of heat shock proteins (HSP) [2–5], although at present, the precise functional role of this expression is uncertain. Tumour cell HSP expression is considered important in the immune response to cancer, possibly *via* enhanced immunological recognition [6, 7]. Notwithstanding this, in the recent studies of Ciocca *et al.* [4] and Kimura *et al.* [5], patients with breast or ovarian carcinoma expressing low levels of HSP demonstrated significantly better survival rates than patients with tumours expressing high levels of HSP. It appears that HSP expression may reflect the degree of tumorigenicity of malignant cells and thus may provide additional information concerning the likely behaviour of oral epithelial malignancies.

Mutant protein products of tumour suppressor genes, such as p53, and oncogenes, such as c-*myc* have been reported in oral cancer and adjacent dysplasia [8–10]. Of interest in the current context is that the 70-kDa HSP (HSP 70) forms oligomeric complexes with p53 and c-*myc* in malignantly transformed cells [11–19]. HSP 70–p53 complexes are believed to extend the half-life of mutant p53 proteins, which may explain the accumulation of these proteins in tumour cells [20]. HSP 70–c-*myc* complexes, on the other hand, may inhibit tumour cell cycling, resulting in enhanced tumour cell differentiation [21]. Hence, p53 and c-*myc* abnormalities, described in oral epithelial dysplasia and carcinoma, may be associated with aberrant HSP 70 expression in these lesions. Accordingly, the aim of the present study was to examine HSP 70 expression in benign, dysplastic and malignant lesions of the oral mucosa. In this study, HSP 70 expression is shown to be increased in benign,

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Manuscript received 15 Feb. 1994; manuscript accepted 19 Sep. 1994.

dysplastic and malignant lesions compared with normal oral mucosa. The significance of this expression is discussed.

MATERIALS AND METHODS

Tissue specimens

Lesional tissue was available from 29 patients with oral squamous cell carcinoma (SCC), 18 patients with oral epithelial dysplasia and 22 patients with benign oral mucosal lesions. The SCCs were graded as well differentiated ($n=14$), moderately differentiated ($n=10$) and poorly differentiated ($n=5$). The dysplastic oral lesions were graded as mild dysplasia ($n=10$) or moderate dysplasia ($n=8$). The benign lesions included fibroepithelial polyps ($n=15$) and fibrous epulides ($n=7$). Normal oral mucosa from 15 healthy subjects was used for comparison. Sections of normal tonsil were used as positive controls for HSP 70 expression. All tissue was formalin-fixed paraffin-embedded archival material. Hence, variations due to differences in fixation were not controlled. Diagnoses were confirmed by the same pathologist following re-examination of haematoxylin and eosin-stained sections from the recut blocks.

Immunohistochemistry

From each block, three 4-μm sections approximately 40 μm apart were deparaffinised, rehydrated, washed and treated with a solution comprising 2% horse serum and 0.1% bovine serum albumin containing 0.1% sodium azide in 150 mmol/l phosphate-buffered saline (PBS; pH 7.2) for 15 min to block non-specific antibody binding. The primary antibody was a polyclonal rabbit anti-HSP 70 antibody (Dako Corporation, Carpinteria, California, U.S.A.) specific to heat shock protein (DnaK) from *E. coli*, which shares greater than 48% sequence homology with mammalian HSP 70 [22, 23]. This anti-HSP 70 antibody recognises both the constitutively-expressed 73-kDa member of the HSP 70 family (HSP 73) and the stress-inducible HSP 72 [24]. HSP 72 and HSP 73 are nuclear and cytoplasmic proteins that exhibit approximately 95% sequence homology [25]. It was shown recently that HSP 72 and HSP 73 combine to form a stable complex in stressed cells [26]. The optimal dilution of anti-HSP 70 antibody (1:500) was determined by titration. Sections were incubated with diluted primary antibody for 2 h at room temperature (RT). The second layer consisted of biotin-conjugated donkey anti-rabbit immunoglobulin (Amersham International, Arlington Heights, Illinois, U.S.A.) diluted 1:200 in PBS. Incubation was for 30 min at RT. The third layer was an avidin–biotin–horseradish peroxidase complex (Vectastain Elite, Vector Laboratories, Burlingame, California, U.S.A.) diluted 1:50 in PBS. Incubation was for 30 min at RT. Sections were washed for 10 min in two changes of PBS between each layer. The colour reaction was developed with a solution comprising 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma Chemical Co., St Louis, Missouri, U.S.A.), hydrogen peroxide (0.01%) and nickel chloride (0.03%) in 48 mmol/l Tris–HCl (pH 7.6). Counter staining was with Mayer's haematoxylin. Non-specific binding of the second and third layers was controlled by the omission of the preceeding layer. To control for non-specific binding of the primary antibody, non-immune rabbit serum, at the same final protein concentration as the rabbit anti-HSP 70 antibody preparation, was substituted as the first layer for staining of serial sections.

Evaluation of sections

Sections were coded and mixed to ensure a blind protocol. The procedure for electronic image analysis is a modification of a previously published method [27, 28]. Using a projection microscope (Zeiss 477810; Carl Zeiss, Oberkochen, Germany), sections were imaged under standardised conditions on to a vertical numbered grid at a final magnification of × 1000. Light intensity in the epithelial compartment was measured using a purpose-built microphotodensitometer at 10 randomly-selected points on the grid. The photoresistor sensor was circular, and when adjusted for final magnification, the diameter of the sensor was 5 μm. The sensor was placed over the image of the stained cells and the resistance recorded in kilo-ohms (kΩ). Background light intensity due to the glass slide, mountant and cover slip was measured at the four corners and the centre of the grid. The distribution of epithelial HSP 70 expression was assessed concurrently.

Statistical analysis

Background light intensity was subtracted from each measurement. The staining intensity value for each block was the mean of 30 measurements. Densitometry data were analysed non-parametrically using Kruskall–Wallis (incorporating Newman–Keuls multiple comparisons) and Mann–Whitney tests. Distribution data were analysed by two-tailed Fisher's exact test. Differences were considered significant at P values less than 0.05.

RESULTS

Distribution of HSP 70 expression

Almost one half of the SCCs (14/29; 48%) demonstrated an even distribution of HSP 70 expression by epithelial cells throughout the tumour. This staining pattern accounted for 8/14 (57%) of the well differentiated and 6/10 (60%) of the moderately differentiated SCC lesions. None of the five poorly differentiated SCCs demonstrated this staining pattern. When analysed with Fisher's exact test, the staining distribution in well differentiated SCC was significantly different from that in poorly differentiated SCC ($P<0.05$). In the remainder of the SCCs, epithelial tumour cell staining was focal in distribution and there was occasional increased staining in the "basal" epithelial layer (Figs 1–3). Approximately two thirds of the dysplastic oral mucosal lesions (11/18; 61%) demonstrated negative basal layer staining, positive staining in the deep prickle layer, a relatively unstained band beneath the keratin layer and variable surface staining. This staining pattern was present in 6/10 (60%) of the mildly dysplastic and 5/8 (63%) of the moderately dysplastic lesions. There was no significant difference in the distribution of HSP 70 expression between these groups (Figs 4, 5). In the remainder of the dysplastic lesions, the staining distribution was similar to that seen in the benign lesions and normal oral mucosa. The staining distribution in the benign lesions (Fig. 6) was, in all 22 cases, similar to that seen in normal oral mucosa (Fig. 7). This pattern was characterised by staining throughout all epithelial layers, with occasional weak staining in the basal layer and increased staining in the prickle layer.

Intensity of HSP 70 expression

The staining intensity of the positive control (normal tonsil with anti-HSP 70 antibody as the primary layer) was 11.6 kΩ (Fig. 8). The staining intensity of the negative control (normal

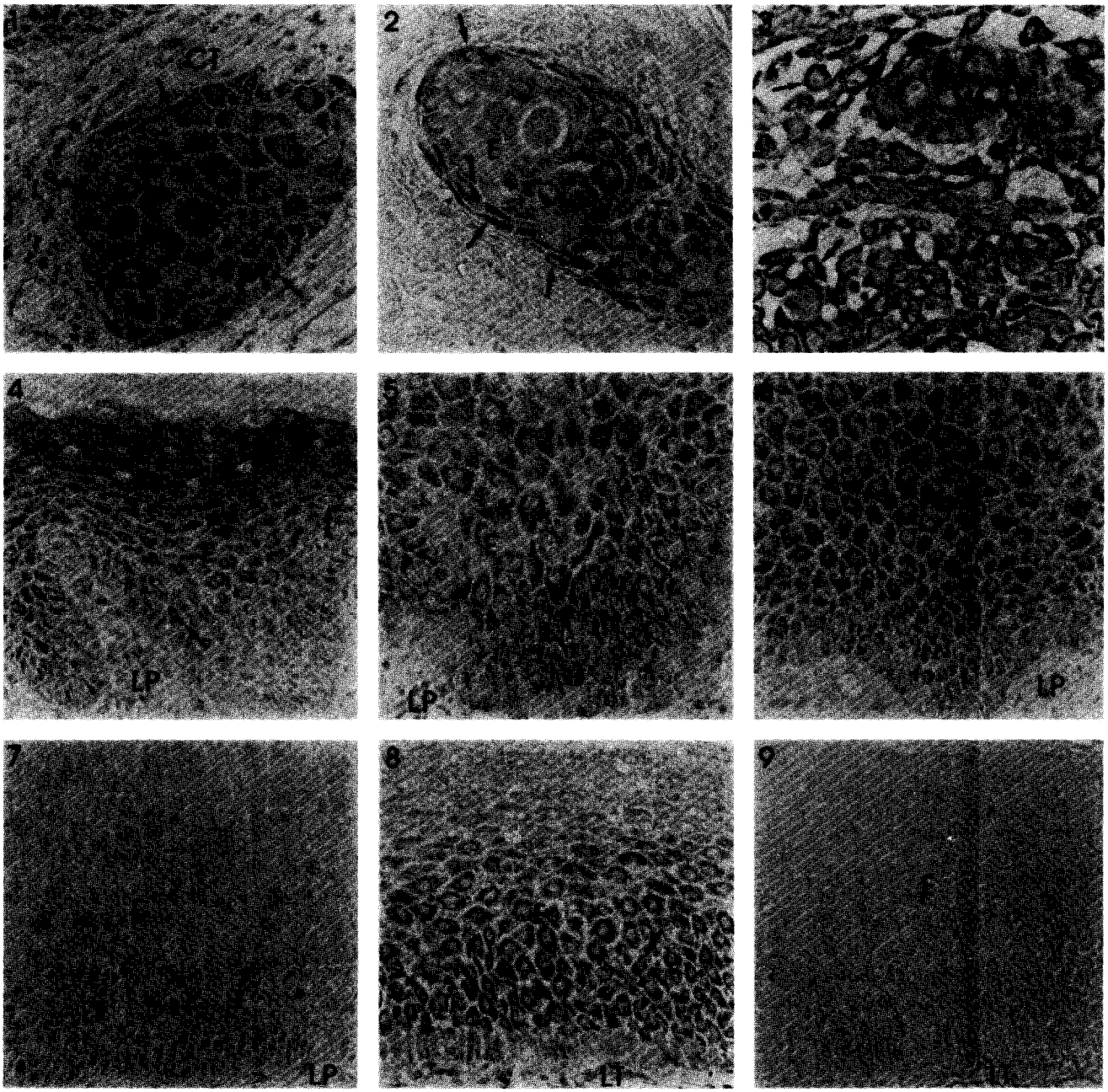

Fig. 1. HSP 70 expression in well differentiated oral squamous cell carcinoma (SCC). This section demonstrates even staining distribution within the epithelium of the tumour. Note the slightly increased staining intensity in the "basal" layer of this particular epithelial island.

Fig. 2. HSP 70 expression in moderately differentiated oral SCC. This section demonstrates focal staining distribution within the epithelium of the tumour.

Fig. 3. HSP 70 expression in poorly differentiated oral SCC. This section demonstrates an intensely stained focus of tumour cells.

Fig. 4. HSP 70 expression in mild oral epithelial dysplasia.

Fig. 5. HSP 70 expression in moderate oral epithelial dysplasia. Note the relatively unstained basal epithelial layer and the intense staining in the deep prickle layer.

Fig. 6. HSP 70 expression in a fibrous epulis. Note the even distribution of staining throughout the epithelium.

Fig. 7. HSP 70 expression in normal oral mucosa. The intensity of HSP 70 expression in this section of normal oral mucosa is less than that in oral SCC (Figs 1–3), oral epithelial dysplasia (Figs 4, 5) and benign oral mucosal lesions (Fig. 6).

Fig. 8. HSP 70 expression in tonsillar epithelium (positive control).

Fig. 9. Tonsillar epithelium with non-immune rabbit serum substituted as the primary layer (negative control). Sections in Figs 1–8 were stained with polyclonal rabbit anti-HSP 70 antibody, as described in the text. E, epithelium; CT, connective tissue; LP, lamina propria; LT, lymphoid tissue; arrows = epithelial cells demonstrating anti-HSP 70 immunoreactivity. Final magnification × 150.

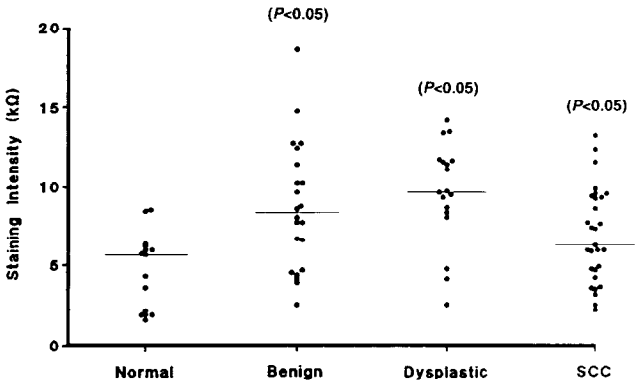

Fig. 10. Results of microdensitometric analysis of HSP 70 expression in normal oral mucosa, benign oral mucosal lesions, dysplastic oral mucosa and oral squamous cell carcinoma (SCC). Staining intensity was measured in kilo-ohms (kΩ), as described in the text. Each datum point is the mean of 30 readings from each block. Bars are group medians. Figures in parentheses are levels of significance of the differences in staining intensity when each group was compared (Kruskall–Wallis) with normal oral mucosa.

tonsil with non-immune rabbit serum as the primary layer) was 2.3 kΩ (Fig. 9). Microdensitometry results are presented in Table 1 and Fig. 10. The median staining intensity and range in normal oral mucosa (5.64 kΩ; 1.49–8.45 kΩ) was significantly less than that in SCC (6.22 kΩ; 2.13–13.22 kΩ), epithelial dysplasia (9.61 kΩ; 2.41–14.09 kΩ) and the benign oral mucosal lesions (8.28 kΩ; 2.45–18.45 kΩ), with P values in all cases less than 0.05. There was no significant difference in the staining intensity between SCC, epithelial dysplasia and the benign oral mucosal lesions. Median staining intensity in poorly differentiated SCC (7.66 kΩ; 5.93–12.33 kΩ) was greater than that in moderately differentiated SCC (4.77 kΩ; 2.13–11.56 kΩ), although this result just failed to reach statistical significance ($P=0.06$). There was no significant difference in the staining intensity between poorly differentiated and well differentiated SCC (6.75 kΩ; 2.44–13.22 kΩ). There was no significant difference in the staining intensity between mild (10.39 kΩ; 4.74–14.09 kΩ) and moderate (9.61 kΩ; 2.41–13.45 kΩ) epithelial dysplasia.

DISCUSSION

Previous studies have shown that tumour cells, both *in vivo* and *in vitro*, express elevated levels of HSP [2–5]. It is believed that tumour cell HSP expression is important in the immune response to cancer [6, 7]. In addition, it was shown that patient survival in breast and ovarian carcinoma may correlate with the level of HSP expression in these tumours [4, 5]. Accordingly, the current investigation examined HSP 70 expression in benign, dysplastic and malignant lesions of the oral mucosa. Results show that the distribution of HSP 70 expression in poorly differentiated SCC (Fig. 3) was significantly different ($P<0.05$) from that in well differentiated SCC (Fig. 1), the former demonstrating a more focal staining pattern. Secondly, the intensity of HSP 70 expression was greater ($P<0.05$) in dysplastic (Figs 4, 5) and malignant (Figs 1–3) epithelial lesions than in normal oral epithelium (Fig. 7). Thirdly, HSP 70 expression in poorly differentiated SCC (Fig. 3) was greater than that in moderately differentiated SCC (Fig. 2), although this result just failed to reach statistical significance ($P=0.06$). Thus, it appears that HSP 70 expression has potential significance in investigations of oral epithelial dysplasia and squamous cell carcinoma.

These results must be considered with the caveat that markers of malignancy or potential malignancy may reflect increased cell proliferation rather than a state or stage of malignant transformation [1]. In this context, it was shown previously that HSP 70 has a role in cell replication and proliferation [29, 30]. However, in the current investigation this seems unlikely, as epithelial HSP 70 expression was increased significantly in both malignant and dysplastic lesions, while in the latter, this expression was not coincident with obvious epithelial thickening (Fig. 4). The staining distribution in the benign lesions (Fig. 6) was similar to that seen in normal oral mucosa (Fig. 7), although the staining intensity was significantly greater in the former. As HSP expression can be induced by a diverse range of non-lethal cellular stresses [25, 31], increased HSP 70 expression in the benign lesions may be attributable to the degree of mechanical trauma to which these lesions were subjected *in vivo*.

It is generally considered that oral epithelial lesions with more severe dysplasia have greater malignant potential. In the current investigation, the dysplastic lesions were graded as mild or moderate. There was no significant difference in HSP 70 staining intensity or distribution between these groups. Similarly, there was no significant difference in HSP 70 staining intensity between oral epithelial dysplasia and SCC. Although increased HSP 70 expression was evident in the dysplastic and malignant oral lesions compared with normal oral mucosa, there

Table 1. Intensity of HSP 70 expression in normal oral epithelium, benign oral mucosal lesions, dysplastic oral epithelium and oral squamous cell carcinoma (SCC) (kΩ)

			Dysplastic			SCC			
	Normal	Benign	Mild*	Moderate*	Total	Well†	Moderate†	Poor†	Total
n	15	22	10	8	18	14	10	5	29
Median	5.64	8.28	10.39	9.61	9.61	6.75	4.77	7.66	6.22
Range	1.49–8.45	2.45–18.55	4.74–14.09	2.41–13.45	2.41–14.09	2.44–13.22	2.13–11.56	5.93–12.33	2.13–13.22

The median staining intensity in normal oral epithelium was significantly less than that in SCC, epithelial dysplasia and the benign oral mucosal lesions with P values in all cases less than 0.05. There was no significant difference in the staining intensity between SCC, epithelial dysplasia and the benign oral mucosal lesions. There was no significant difference in the staining intensity between mild and moderate epithelial dysplasia. Median staining intensity in poorly differentiated SCC was greater than that in moderately differentiated SCC, although this result just failed to reach statistical significance ($P=0.06$). There was no significant difference in the staining intensity between poorly differentiated SCC and well differentiated SCC. * Severity of dysplasia. † Degree of differentiation.

was also increased expression in the benign oral mucosal lesions, suggesting that HSP 70 expression lacks specificity. Furthermore, oral cancer cells produce altered patterns of cytokeratins and other protein products [1]. As HSP 70 is involved in protein synthesis, folding and translocation [32–34], increased HSP 70 expression in oral SCC may be merely an epiphenomenon related to the synthesis of a variety of proteins by the tumour cells, rather than a marker of malignancy *per se*.

In conclusion, the results of the current investigation demonstrate increased HSP 70 expression in benign, dysplastic and malignant lesions of the oral mucosa. These results suggest that, as employed currently, HSP 70 expression is not a definitive or specific marker of oral malignancy or malignant potential. However, HSP 70 is implicated in the degree of tumour differentiation [21], the rate of tumour proliferation [20] and the magnitude of the anti-tumour immune response [6, 7]. Tumour HSP 70 expression may, therefore, have profound biological and prognostic significance. Hence, with further development, quantitative analysis of anti-HSP 70 immunoreactivity in formalin-fixed, paraffin-embedded tissue may be valuable as an adjunct to conventional histology for assessing the malignant potential of oral mucosal lesions. Further work is required to determine whether levels of HSP 70 expression in oral epithelial dysplasia and carcinoma correlate with the intensity of the local immune response and with clinical outcomes.

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Acknowledgements—This investigation was supported in part by the Queensland Cancer Fund, the National Health and Medical Research Council of Australia and the Australian Dental Research Fund, Incorporated.