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Absence of Retinoblastoma Gene Product in Human Primary Oral Cavity Carcinomas*

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Oral cavity cancer is a major health concern worldwide. Despite advances in surgery, radiotherapy and chemotherapy over the past 35 years, there has been no significant enhancement in the survival of oral cavity cancer patients. Improved survival will require identification of reliable prognostic markers that provide a rational basis for assessment of risk for progression. The altered retinoblastoma (RB) gene has been linked to the hereditary retinoblastoma. This gene is defective in several types of human malignancies. The intent of this study was to evaluate the role of the RB gene in oral cavity tumorigenesis and to explore whether or not there is a relationship between the loss of RB protein and each of several clinicopathological parameters in oral cavity carcinomas. We have analysed the expression of the RB gene in four cell lines (J82, ML1, SaOS2 and WERI-RB-1), 182 oral cavity carcinomas (75 T1 and 107 T3 and T4 lesions) and 55 normal tissues adjacent to cancer by means of an immunohistochemical method and Western immunoblotting. The expression of RB protein was then correlated with clinical outcome in the patients with primary tumours. The significantly higher rate of altered RB expression was found in advanced oral cavity tumours (40 of 107; 37%) in comparison with low grade tumours (9 of 75; 7%). In T3 and T4 tumours, RB gene expression did not correlate with presence or absence of lymph node metastasis, degree of differentiation and patient survival. However, in the T1 cohort, poorer survival rate was seen for those patients who had a tumour with loss of RB protein. This study suggests that tumours in which the RB protein was altered were more aggressive than tumours in which the RB protein was present and that loss of RB protein in oral cavity cancer may be a prognostic variable of tumour progression.

Key words: oral cavity cancer, retinoblastoma gene product, immunohistochemistry, prognosis

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INTRODUCTION

Oral cavity cancer is a major health concern among males and is the predominant cause of cancer death in some regions of Asia and Africa. In an attempt to better understand the molecular biology of head and neck cancer, recently we [1, 2] and others [3] for the first time demonstrated that the retinoblastoma (RB) gene has been altered in this disease. Aberrant RB gene has been linked to the hereditary retinoblastoma [4] and there is growing evidence that the defective RB gene might play a role in tumorigenesis of a diverse group of human malignancies such as carcinomas of the lung [5, 6], breast [7], bladder [8], prostate [9], colorectum [10], testis [11], ovary [12], kidney [13], oesophagus [14], cervix [15], liver [16], malignant gliomas [17], sarcomas [18] and leukaemias [19, 20]. Underphosphorylated RB protein is a negative regulator of the cell cycle prohibiting progress from mid-G1 into S phase [21]. Phosphorylation of RB protein is required for transition into S phase and might be triggered by activated cyclin–CDK complexes. During G1 phase of the cell cycle, RB protein is bound to the transcription factor E2F, but upon phosphorylation E2F is released and activates the transcription of genes required for transition into S phase [22]. Since an anti-RB protein monoclonal antibody is now

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available for formalin-fixed, paraffin-embedded tissue sections, the study was undertaken to evaluate the role of the RB, gene in oral cavity carcinogenesis.

MATERIALS AND METHODS

Case selection

Paraffin blocks from 182 patients with primary squamous cell carcinoma of the oral cavity were obtained from the University of Cincinnati and the Veterans Administration, U.S.A. Medical Centers, Cincinnati, Ohio, U.S.A. Of the specimens examined, 75 were diagnosed as T1 and 107 as T3 and T4, respectively. All 55 normal tissues used for comparison were adjacent to cancerous lesions. All patients included in this study had an initial single primary lesion and none had undergone any pre-operative treatment. The minimum follow-up was 17 months for 46 patients who were disease free (no evidence of disease). 16 patients died of oral cancer and 13 died of unrelated illnesses.

Immunohistochemistry

Tissue fixed in 10% neutral buffered formaldehyde and processed for routine surgical pathology evaluation were cut as 5 μm sections and mounted on polylysine-coated slides. A method for revealing previously inaccessible epitopes in paraffin-embedded tissues was described by Shi *et al.* [23] and modified by Pavelic *et al.* [24]. In brief, this procedure entails microwave heating of tissue sections to 100°C in the presence of a metal solution. Specifically, the tissues were deparaffinised and rehydrated by standard methods, the endogenous peroxide activity was blocked with 3% hydrogen peroxide, and the slides were washed for 5 min with distilled water. The slides were placed in Coplin jars containing a 1% zinc sulphate solution and heated for 10 min in a microwave oven at the higher power setting. After cooling for 15 min, the slides were rinsed in distilled water and phosphate-buffered saline (PBS) and treated for 6 min with 2.5% trypsin. The application of trypsin was essential for RB epitope retrieval. The mouse anti-RB monoclonal antibody (Clone LM95.1, a gift from Oncogene Science Inc., Cambridge, Massachusetts, U.S.A.) was used at a dilution of 1:10 (10 μg/ml) for staining RB protein. The clone 2A8 was derived by immunisation of BALB/c mouse with recombinant RB corresponding to the carboxyterminal 56 kDa fragment and fusion of its spleen cells with NS1 mouse myeloma cells [25]. According to the manufacturer, this antibody was purified from mouse ascites fluid by nondenaturing liquid chromatography and reacts with the RB p105 protein in human and mouse tissue, but not with p107 protein. The epitope has not been determined but is in the C-terminal portion of the RB protein. Immunolocalisation was carried out using the peroxidase–antiperoxidase (PAP) method described elsewhere [26]. Diamino-benzidine tetrahydrochloride (DAB) and amino ethyl carbazol (AEC) were used as the chromogen and Harris haematoxylin as the nuclear counterstain. As a negative control, tissues were incubated with PBS instead of primary antibody before addition of the peroxidase-coupled secondary antibody. The J82 cell line derived from a human poorly-differentiated, invasive, stage T3 transitional cell carcinoma of urinary bladder [27] and human myeloblastoma cell lines (ML1) were used as a positive control for RB expression. The human retinoblastoma WERI-RB-1 and osteogenic sarcoma (SaOS2) cell lines which lack RB gene expression served as negative controls. All cell lines were obtained from the American Type Culture Collection, Rockville, Maryland, U.S.A. Twenty-three tumour samples were obtained in the operating room immediately following the resection of the primary neoplasm and processed within 10–20 min after surgical removal. The specimens were placed in OTC (Miles Inc, Elkhart, Ind.) and frozen in dry ice. The tissues were stored at −70°C until sectioned. Prior to staining, the frozen tissues were cut at 5 μm thickness, fixed in 95% cold (4°C) acetone, and air dried overnight. These samples were used for comparison of RB staining with microwave-heated tumour sections.

Evaluation of slides

For each tissue specimen a pathologist (Z.P.P.) and otorhinolaryngologist (M.L.) determined the fraction of stained cells. A tumour was considered to be RB positive if any of its malignant cells had RB nuclear staining. Tumours were

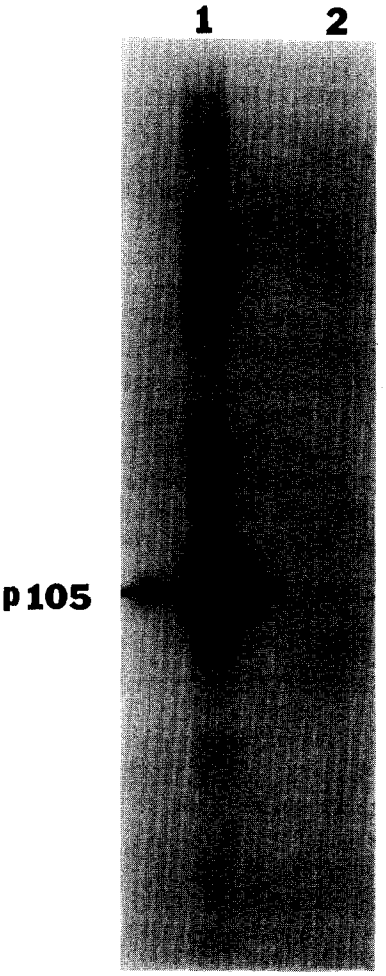

Fig. 1. Lysates were made from the Rb-positive human myeloblast cell line, ML1 (top) or the Rb-negative human osteogenic sarcoma cell line, SaOS2 (bottom) and applied to a 10% SDS–PAGE gel. The resolved proteins were transferred to nitrocellulose and probed with the anti-Rb monoclonal, LM95.1 (Rb (Ab-5)) at concentrations of 1 μg/ml, 0.5 μg/ml, and 0.1 μg/ml and detection was performed using chemiluminescence.

Table 1. Comparison of RB protein staining among frozen, conventional, and microwave-heated oral tumour tissue sections

	Staining intensity* (No. (%) of cases)				
	–	+	+ +	+ + +	Total
Frozen	8 (35)	3 (13)	7 (30)	5 (22)	23
Microwave oven	8 (35)	4 (17)	8 (35)	3 (13)	23
Conventional oven	14 (61)	6 (26)	3 (13)	0	23

* –, no staining; +, 1–33% positive nuclei; + +, 34–66% positive nuclei; + + +, 67–100% positive nuclei.

judged to be negative only if all malignant cells failed to stain for Rb nuclear protein.

Western immunoblots

Cell lysates from the RB positive cell line ML1 (human myeloblast) and the RB negative cell line SaOS2 (human osteogenic sarcoma) [1] were prepared from exponentially growing cell line cultures and then processed for direct Western immunoblotting as described previously [28]. Each lane of the gel was loaded with 20 µl of cell lysate corresponding to 20 µg total cellular protein.

Table 2. RB protein expression in normal and tumour tissues from the oral cavity

		RB protein expression		
Group	No. of cases	Normal (%)	Altered* (%)	*P*-value
Normal	55	55 (100)	0	
T1	75	68 (91)	7 (9)	
T3 and T4	107	67 (63)	40 (37)	0.05

* Lack of RB protein in every tumour cell.

Statistical analysis

The chi-square and Fisher exact T tests were used for statistical evaluation of the results.

RESULTS AND DISCUSSION

Expression of RB protein (p105) was demonstrated by Western immunoblotting in established human myeloblast cell line ML-1 but not in the SaOS2 osteogenic sarcoma cell line which lacks the RB gene (Fig. 1). This data confirmed the immunocytochemical studies and the specificity of the antibody. We did not detect cross-reacting RB-related proteins in the 107 kDa size range. This antibody, therefore, has been chosen in our laboratory to develop a highly specific immunohistochemical staining method.

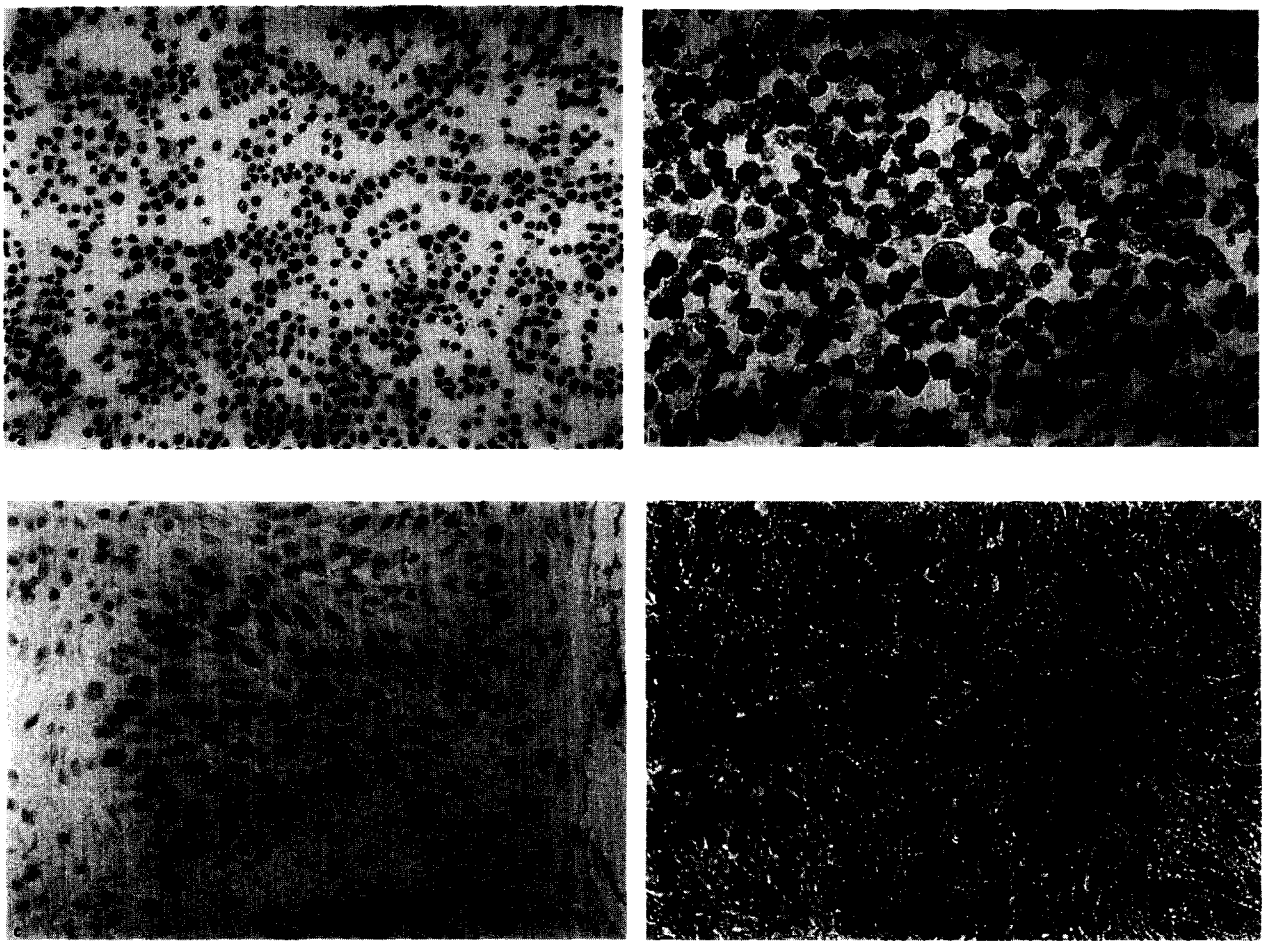

Fig. 2. Immunohistochemical staining of RB protein in cell lines and primary oral cavity carcinomas, using archival paraffin-embedded tissue sections. (A) RB positive J82 cell line. Note nuclear staining. (B) RB negative human retinoblastoma cell line, WERI-RB-1. (C) RB positive early stage T1 oral cavity cancer. RB is detectable in the nuclei of tumour cells. (D) RB negative late stage T4 oral cavity cancer (× 150).

The comparative efficacy of different tissue-processing for RB protein using immunohistochemistry was assessed in sections from 23 oral cavity carcinoma tissue samples (Table 1). The data demonstrate that RB protein was equally detected in frozen (15/23) and 10% buffered formalin-fixed, paraffin-embedded (15/23) tumour samples, after using the antigen retrieval method and application of trypsin. This study demonstrated that the epitope recognised by Ab-4 antibody is well preserved in tumour and normal tissues fixed in 10% buffered formalin. However, certain alterations in the RB gene might alter the epitope recognised by Ab-4 or render it inaccessible.

One hundred and eighty-two oral cavity carcinomas and 55 adjacent normal tissues were analysed for presence or absence of RB protein (Table 2). The heterogeneous nuclear staining pattern (positively and negatively stained tumour cells throughout the section) was observed in the majority of tumour samples and may reflect the fact that the level of normal RB protein is cell-cycle dependent [3]. Examples of RB+ and RB − cases as measured by immunohistochemistry are shown in Fig. 2. The majority of normal cells, such as lymphocytes, endothelial cells, and stromal cells of the 55 normal tissue samples demonstrated RB-positive nuclear staining (Table 2). Nine of 75 (7%) T1 and 40 of 107 (37%) T3 and T4 lesions were found to have negative RB staining (Table 2). The higher rate of altered RB expression found in advanced oral cavity tumours strongly suggests that loss of RB protein might be associated with tumour progression in this malignancy. The molecular basis for the absence of RB protein expression in T3 and T4 oral cavity cancer patients is not yet known. However, preliminary data from our laboratory demonstrated the loss of heterozygosity on chromosome 13q14 in 15% of head and neck cancer patients [21]. Yoo *et al.* [3] reported infrequent inactivation of the RB gene despite frequent loss of chromosome 13 in head and neck cancer patients.

To explore whether or not there is a relationship between the absence of RB protein and each of several clinicopathological parameters in T3 and T4 oral cavity cancer, immunostaining data were scored as a function of each of the parameters. The parameters evaluated included tumour stage, presence or absence of lymph node metastasis, degree of differentiation, and patient survival. The data (not shown) indicate a lack of significant correlation between absence of RB protein in tumour cells and any of the parameters examined, and suggest that in this group of patients functional loss of RB protein might be an independent factor in tumour progression [5, 15]. However, the RB protein expression in T1 oral cavity cancer correlated with clinical course and survival of these patients. Of 30 alive patients, 2 (7%) demonstrated absence of RB protein in tumour samples (Table 3). In contrast, five of 13 (38%) T1 lesions from a cohort of dead patients demonstrated negative RB immunostaining. Six of seven RB negative T1 tumours deemed aggressive based on their propriety for regional or distant metastasis or local aggressive histopathology at the primary site and, of these, five (83%) belong to patients who died (Table 4). Survival was expressed as the number of months from the date of primary surgery to the date of death if due to the malignancy. Since the tumours in which the expression of RB gene was lost were more aggressive than tumours in which the protein was present and since no important differences in prognostic characteristics were detected between patients in the two groups, we postulated that loss of RB protein may be an important prognostic marker for early oral cavity cancer. Consistent with this theory is the fact that all alive patients had normal RB expression in their tumour cells. Patients with altered RB protein may be candidates for more aggressive therapy. Because of the small number of patients in this cohort the conformation of these data by other researchers would support this recommendation.

Table 3. Comparison of RB protein expression in T1 oral cancer with survival

	Number of cases	Altered RB	Normal RB	*P*-value
Alive	30	2	28	
Dead	13	5	8	0.05
No follow-up	32	0	32	

Table 4. Comparison of altered RB protein expression in T1 oral cancer with histopathology and survival

Patient no.	Histopathology	Survival (months)
1	Not aggressive	98
2	Aggressive	43
3	Aggressive	Dead
4	Aggressive	Dead
5	Aggressive	Dead
6	Aggressive	Dead
7	Aggressive	Dead

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