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RESEARCH

Ribosomal Protein S6 Phosphorylation is Associated with Epithelial Dysplasia and Squamous Cell Carcinoma of the Oral Cavity

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Abstract Ribosomal protein S6 (RPS6), a downstream effector of the mammalian target of rapamycin pathway (mTOR), is activated in many cancers including oral squamous cell carcinoma (OSCC). However, the role of RPS6 in the progression of potentially malignant disorders (or premalignant lesions) to OSCC is unknown. The purpose of this study was to examine the expression of RPS6 in epithelial dysplasia and OSCC to determine the association of RPS6 in tumor progression. In our study, an immunohistochemical analysis of RPS6 was performed on tissue microarrays containing 30 control samples, 15 epithelial dysplasia cases, and 53 OSCC cases. Correlations between the clinicopathologic features of OSCC and RPS6 expression were analyzed using the Chi-square test. We found RPS6 phosphorylation (p-RPS6) in 15/30 (50 %) control normal oral mucosa samples, 15/15 (100 %) epithelial dysplasia cases, and 47/53 (88.68 %) OSCC cases. The frequency of p-RPS6 in epithelial dysplasia or OSCC showed a statistically significant difference compared to control ($P<0.001$). However, there were no significant correlations between p-RPS6 and the clinicopathologic features of OSCC. Our findings suggest that RPS6 activation is associated with the early events of tumor progression, suggesting p-RPS6 as a potential marker for early detection of oral cancer.

Keywords Ribosomal protein S6 · Epithelial dysplasia · Oral squamous cell carcinoma · Tumor progression

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Abbreviations

OSCC	Oral squamous cell carcinoma
PMD	Potentially malignant disorders
RPS6	Ribosomal protein S6
S6K	Ribosomal protein S6 kinase
TOP mRNAs	5' terminal oligopyrimidine tract of mRNA
mTOR	Mammalian target of rapamycin
PI3K/AKT	Phosphatidylinositol-3-kinase/ protein kinase B

Introduction

Oral squamous cell carcinoma (OSCC), the most common type of malignancy in the oral cavity, is a serious worldwide health problem. Despite great advances in the diagnosis and treatment of OSCC, the mortality rate for patients with OSCC has increased in most parts of the world. In addition, a high proportion of patients present to the physician with advanced stage disease, resulting in poor outcomes after treatment [1, 2]. OSCC may develop in the clinical presence of potentially malignant disorders (PMD) with oral leukoplakia being the most frequent PMD associated with the progression to OSCC [1, 3]. Indeed, one third of the patients who present with oral leukoplakia that is diagnosed as epithelial dysplasia, the lesion subsequently develops into OSCC [4]. Patients who are diagnosed at an early stage of the disease have a high chance of being cured with a functional outcome [2]. Thus, a molecular test to assess the malignant progression risk in epithelial dysplastic lesions or to detect early stage OSCC may help to decrease patients' morbidity and mortality.

Ribosomal protein S6 (RPS6), the downstream substrate of ribosomal protein S6 kinase (S6K), is involved in the

regulation of cell proliferation, cell growth, and protein synthesis. RPS6 plays an important role in protein synthesis. The phosphorylation of RPS6 by S6K increases the affinity of the ribosome for a 5' terminal oligopyrimidine tract of mRNA (TOP mRNAs). This facilitates the translation initiation of this class of mRNA [5, 6]. Evidence shows that mice, or various cell types, deficient in RPS6 showed reduced cell growth and cell proliferation, suggesting RPS6 functions in the control of cell proliferation and cell growth [7, 8]. RPS6 is highly expressed in various cancers including head and neck squamous cell carcinoma, making it an attractive molecular marker to predict the malignant progression of lesions [9, 10]. The aim of this study was to determine the expression of RPS6 in normal oral mucosa, epithelial dysplasia, and OSCC, to determine the initiation point of RPS6 expression during tumor progression.

Materials and Methods

The study protocol was approved by the Ethics Committee of Chulalongkorn University.

Tissue Collection and Tissue Microarray Construction

A total of 98 paraffin-embedded tissue blocks comprising normal oral mucosa (30 cases), epithelial dysplasia (15 cases), and OSCC (53 cases) were obtained from the Department of Oral Pathology, Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand. The suitability of the tissue blocks for tissue microarray construction was evaluated using criteria described by Molinolo AA et al. [10]. Hematoxylin and eosin stained sections of each case were examined to determine the suitability of the tissue to be included in the study. Two blinded board-certified oral pathologists (RC and SR) confirmed the histological diagnosis of all cases. Patient records on age, sex, lesion location and histologic grading were collected.

Tissue microarrays were constructed utilizing a manual tissue arrayer-1 (Beecher Instruments, Silver Spring, MD, USA) according to a previously described protocol by Kanonen J et al. [11]. Two tissue cores, 1 mm in diameter, were taken from representative areas of each paraffin-embedded tissue block to assemble the arrays. Serial five-μm-thick sections of tissue array blocks were cut and one hematoxylin and eosin stained slide was made to verify the presence of a diagnostic lesional area.

Immunohistochemistry

Immunohistochemical examination of RPS6 expression was performed using an anti- phosphorylated-RPS6 antibody (Ser240/244) (p-RPS6) from Cell Signaling Technologies (Beverly, MA, USA) according to the manufacture instructions. Briefly, five-μm-thick sections of paraffin-embedded tissue mounted on glass slides were deparaffinized and rehydrated. The slides were placed in Citra-solution (Biogenex CA, USA) and treated for two cycles. The endogenous peroxidase activity and non-specific protein reaction were blocked. After extensive washing, the sections were incubated with primary antibody diluted at 1:2000 overnight at 4 °C. The sections were then incubated with SignalStain® Boost IHC Detection Reagent from Cell Signalling Technologies (Beverly, MA, USA) for 30 min at room temperature. Diaminobenzidine substrate was added to the sections for a five min incubation. Finally, slides were stained with hematoxylin and mounted with mounting medium.

Scoring at least 10 % positive cells was determined positive in control oral mucosa, epithelial dysplasia and squamous cell carcinoma. Both tissue cores stained positive were considered as a positive case for p-RPS6 activation [10].

Statistical Analysis

Statistical analysis was performed using SPSS for Windows version 13.0 software (SPSS Inc., Chicago, IL, USA). A Chi-square test was employed to assess the statistical significance differences of experimental groups versus control groups. Correlation between p-RPS6 positivity and clinicopathologic parameters were analyzed by Chi-square test. A

Table 1 Subject characteristics

	Epithelial dysplasia (n=15)	OSCC (n=53)
Age (y)		
Mean	58	64
Range	23–79	29–90
Sex		
Female	9 (60 %)	24 (45.28 %)
Male	6 (40 %)	29 (54.72 %)
Site		
Alveolar mucosa/gingiva	2 (13.33 %)	22 (41.51 %)
Buccal mucosa	1 (6.67 %)	7 (13.21 %)
Floor of mouth	1 (6.67 %)	5 (9.43 %)
Tongue	5 (33.33 %)	13 (24.53 %)
Other (lip, palate)	6 (40 %)	6 (11.32 %)
Histologic grading		
Mild	3 (20 %)	
Moderate	4 (26.66 %)	
Severe	6 (40 %)	
Carcinoma in situ	2 (13.34 %)	
Well differentiated		41 (77.36 %)
Moderate-poorly differentiated		12 (22.64 %)

statistically significant difference was considered to be present at $P<0.001$.

Results

The characteristics of the subjects in this study are summarized in Table 1. We found 15 cases of epithelial dysplasia and 53 cases of OSCC. Epithelial dysplasia was identified in subjects who were slightly younger than those with OSCC. The sex and site distribution of epithelial dysplasia and OSCC was relatively similar. The tongue was the most common site for either epithelial dysplasia (33.33 %) or OSCC (24.53 %) to be identified. Epithelia dysplasia was histologically graded as mild, moderate, severe, or carcinoma in situ (CIS). Severe epithelial dysplasia was seen in six cases (40 %), being the most common grade of epithelial dysplasia. OSCC was subclassified based on the differentiation of the tumor cells as well or moderate-poorly differentiated. The majority of the tumors (77.36 %) were well differentiated.

The immunohistochemical study of p-RPS6 was performed using tissue microarray blocks composed of normal oral mucosa, epithelial dysplasia, and OSCC samples. The quality and quantity of staining for p-RPS6 was examined to evaluate the relationship of p-RPS6 to the malignant progression of OSCC. Control normal mucosa showed positive localization of p-RPS6 in 15 of 30 (50 %) cases. In control normal mucosa, p-RPS6 staining was detected solely in the surface epithelium. RPS6 phosphorylation was seen in all epithelial dysplasia cases (100 %) and in 47 of 53 (88.68 %) OSCC cases. The frequency of p-RPS6 positivity in epithelial dysplasia or OSCC showed a statistically significant difference compared to control ($P<0.001$). However, there was no statistically significant difference in p-RPS6 levels between epithelial dysplasia and OSCC. Representative results for p-RPS6 staining in control normal mucosa, epithelial dysplasia and OSCC are shown in Fig. 1. We found

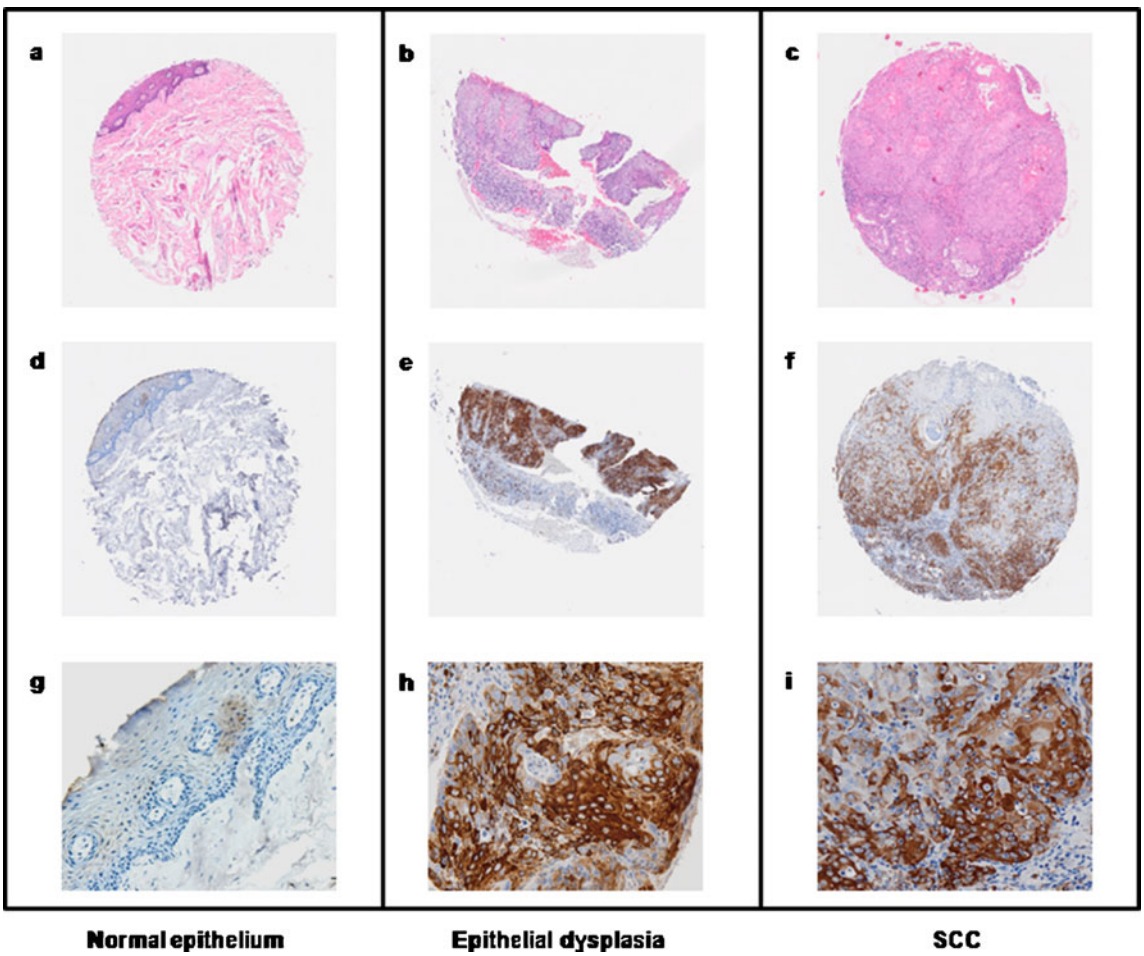

Fig. 1 Immunoreactivity of p-RPS6 in normal epithelium, epithelial dysplasia and squamous cell carcinoma (SCC). Sections of each specimen were stained with hematoxilin and eosin (**a**, **b**, **c**), immunostained for p-RPS6 (**d**, **e**, **f**), and photographed at ×20 magnification (**g**, **h**, **i**). Normal epithelium (**d** and **g**) was negative for p-RPS6 while epithelial dysplasia (**e** and **h**) and SCC (**f** and **i**) demonstrated strongly positive for p-RPS6

p-RPS6 was present primarily in the cytoplasm of the epithelial cells, which was consistent with the RPS6 immunohistochemical profile of other tissues [12, 13]. The pattern of p-RPS6 staining in the majority of the epithelial dysplasia (73.33 %) samples was consistent, showing positive staining in all cell layers of the epithelium. In contrast, positive p-RPS6 staining was limited to the basal cell and parabasal cell layers of the control cases. Notably, positive staining for p-RPS6 was confined to the peripheral zone of the tumor cell nests in the well-differentiated OSCC samples, while the central area of the tumor cell nests consisting of keratinized cells was negative for p-RPS6 staining.

The analysis of any correlations between the clinicopathologic features of OSCC and RPS6 expression are summarized in Table 2. There were no significant associations between p-RPS6 levels and any clinicopathologic features of OSCC.

Discussion

In the present study, we report the results of a tissue-microarray based immunohistochemical analysis of RPS6 localization in oral epithelial dysplasia and OSCC. In addition, the presence of p-RPS6 was evaluated and we analyzed the association of RPS6 phosphorylation with relevant clinicopathological features. Here, we have shown that p-RPS6 was always present in oral epithelial dysplasia (100 %), indicating RPS6 is active in the early events of tumor progression.

Table 2 Immunohistochemical profiles

Lesion	Positive (+)	Negative (−)	*P* value
Epithelial dysplasia (*n*=15)	15	0	–
OSCC (*n*=53)			
Age			0.266 (NS)
<60	15	4	
> = 60, <70	9	3	
> = 70	21	1	
Sex			0.211 (NS)
Male	23	6	
Female	22	2	
Sites			0.396 (NS)
Alveolar mucosa/gingiva	18	4	
Buccal mucosa	6	1	
Floor of mouth	5	0	
Tongue	11	2	
Other (lip, palate)	5	1	
Histologic grading			0.045 (NS)
Well differentiated	37	4	
Moderate-poorly differentiated	8	4	

NS not significant

Phosphorylation of RPS6 is a crucial terminal event of the mammalian target of rapamycin (mTOR) pathway in the regulation of ribosomal biogenesis. The mTOR pathway is activated by a variety of cellular signals, including growth factors, hormones, nutrients, cellular energy levels, and stress. Signaling through phosphatidylinositol-3-kinase/protein kinase B (PI3K/AKT) is the main pathway to activate mTOR, which is a critical mediator of cell survival and proliferation. The activated mTOR pathway mediates the phosphorylation of S6K and RPS6 to regulate ribosomal protein translation and ribosome biogenesis [14, 15]. Studies have revealed that persistent activation of the AKT-mTOR pathway is common in many human malignancies including OSCC [6, 9, 10, 16, 17]. Indeed, a relatively high phosphorylation of RPS6, the terminal event of the AKT-mTOR pathway, has been detected in human head and neck squamous cell carcinoma tissues and cell lines [9, 10, 16]. In our study, we found p-RPS6 in 88.68 % of OSCC cases, supporting prior studies that established the involvement of the deregulated AKT-mTOR pathway in the pathogenesis of OSCC. However, information on the relationship between the status of RPS6 activation and the malignant progression of OSCC is still limited.

Subset of cancers may arise from premalignant lesions. The scheme of malignant progression is thought to be the transformation of normal cells to hyperplastic or dysplastic lesions, to invasive cancers and, finally, metastatic disease. Beginning with the initial efforts to link histopathological changes in colorectal cancer to the mutation of specific genes, progression models have been developed for many cancers [18]. Evidence from experimental models has revealed that OSCC may occur by malignant transformation of hyperplastic or dysplastic lesions into frank invasive carcinomas [19]. Recent studies have evaluated the role of cell cycle regulators, apoptosis regulators, and angiogenesis in the malignant progression of OSCC [20–26]. However, only a few studies have been conducted to determine the role of RPS6 in oral epithelial dysplasia [16]. In our study, p-RPS6 was seen in all oral epithelial dysplasia cases, indicating mTOR activation is involved in oral premalignancy. Indeed, p-RPS6 was detected more frequently in oral epithelial dysplasia (100 %) than in OSCC (88.68 %), suggesting oral epithelial dysplasia is the malignant precursor to a majority of, but not all OSCC cases. The PMDs progressing to the remaining OSCC cases may not involve the mTOR pathway.

In conclusion, our study demonstrated that high levels of p-RPS6 was frequent in oral epithelial dysplasia and OSCC, but was not associated with any clinicopathologic parameters. These findings suggest that the expression profile of

RPS6 could be used as a potential diagnostic marker for early detection of most OSCC.

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