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Up-regulation of a serine–threonine kinase proto-oncogene *Pim-1* in oral squamous cell carcinoma

W.-F. Chiang[1,2,3,4], C.-Y. Yen[1,3], C.-N. Lin[5], G.-A. Liaw[1,2], C.-T. Chiu[6], Y.-J. Hsia[3], S.-Y. Liu[1,3,4]

[1]Department of Oral and Maxillofacial Surgery, Chi-Mei Medical Center, Taiwan; [2]Department of Oral and Maxillofacial Surgery, Chi-Mei Hospital, Liouying, Taiwan; [3]Department of Dentistry, College of Oral Medicine, Taipei Medical University, Taiwan; [4]School of Dentistry, National Defense Medical Center, Taiwan; [5]Department of Pathology, Chi-Mei Medical Center, Taiwan; [6]Department of Oral and Maxillofacial Surgery, Chang Gung Memorial Hospital, Kaohsiung, Taiwan

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Abstract. The *Pim-1* proto-oncogene, encoding a serine–threonine kinase, has been found to play an important role in regulating apoptosis, differentiation, proliferation and tumourigenesis. The present study was conducted to assess the importance of *Pim-1* in oral tumourigenesis *in vivo*. Reverse transcriptase-polymerase chain reaction and immunohistochemistry were used to study the expression of *Pim-1* in oral squamous cell carcinoma (OSCC) and non-cancerous match tissue (NCMT) sampled from the periphery of the tumours. *Pim-1* mRNA expression in OSCC was significantly higher than that in NCMT in 36 tissue pairs (1.33 ± 0.41 versus 0.97 ± 0.29, $P = 0.03$). The percentage of OSCCs exhibiting strong cytoplasmic *Pim-1* immunoreactivity was significantly higher than that of NCMT (60% versus 19%, $P = 0.007$). *Pim-1* immunoreactivity is higher in the more differentiated components of a tumour. In around 10% of OSCC cases, *Pim-1* immunoreactivity was found in the nucleus as well. These results show novel findings of the up-regulation of *Pim-1* expression from NCMT to OSCC. The pathogenetic role of *Pim-1* expression in oral tumourigenesis deserves further investigation.

Key words: oral cancer; *Pim-1*.

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Oral squamous cell carcinoma (OSCC) is a leading cause of cancer deaths worldwide[9]. The prevalence of OSCC in South Asia, which is tightly linked to the popularity of areca chewing, is very high[9,8,16]. The authors' previous study specified that areca activates the Ras-related small GTPase proteins (Rac1 and Rho1) in oral keratinocytes[16]. It was also demonstrated that Rac-1 overexpression, a frequent event of OSCC, highlights the involvement of signaling elements in the neoplastic growth of this tumour[8]. Investigations of additional oncogenomic signatures of OSCC are required to assist the prevention and resolution of oral carcinogenesis.

Pim-1 proto-oncogene is a serine–threonine Pim kinase[15] that has been implicated both in cytokine-induced signal transduction and in the development of lymphoid and prostate malignancies[2,4–6,10]. The up-regulation of *Pim-1* contributes to cellular proliferation, anti-apoptosis activity, differentiation and genomic stability[15]. *Pim-1* and the *myc* oncogene cooperate to promote oncogenesis[2,5]. *Pim-1* kinase enhances Bcl-2 activity and promotes cell survival through phosphorylation of Bad[1]. The concordant expression of *Pim-1* and transglutaminase, a differentiation marker

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of keratinocyte, in cultured epidermis cells has also been shown[11]. Although several substrates of *Pim-1* kinase have been identified[14], the precise role of *Pim-1* in signaling regulation and tumourigenesis remains obscure[15].

In a previous study, a paradoxical role of *Pim-1* in prostate carcinomas was found with an increase in *Pim-1* expression in the vast majority of malignant tissues, while poor survival was correlated with decreases in *Pim-1* expression[4]. To clarify the potential role of *Pim-1* in oral carcinogenesis, the present study assessed *Pim-1* mRNA expression and protein levels in paired oral tissues.

Materials and methods

Samples

Since RNA integrity can be better preserved in frozen tissues than in formalin-fixed tissues, frozen tissue samples were used for mRNA expression analysis[8]. Surgical specimens from 36 OSCCs and their paired non-cancerous match tissue (NCMT) were obtained as approved by an IRB. The OSCC tissues were sampled from resected specimens and frozen immediately in liquid nitrogen until RNA extraction. The ages of the patients ranged from 27 to 72 years (mean: 47 years), and 83% (30 cases) of the patients were areca chewers. The most common primary site was the buccal mucosa (53%, 19 cases) followed by the tongue (25%, 9 cases). With histopathological grading, 33% (12 cases), 56% (20 cases) and 11% (4 cases) showed well, moderate and poor differentiation, respectively; 50% (18 cases) of the patients had lymph-node metastasis and 56% (20 cases) had stage IV OSCC. NCMT was sampled 1 cm away from the resection margin to ensure that there was no contamination by tumour cells[8]. Frozen section evaluation confirmed normal squamous epithelium with focal hyperkeratosis or hyperplasia. No epithelial dysplasia was noted in the NCMT.

Immunohistochemical (IHC) staining was performed on archival formalin-fixed paraffin-embedded tissue sections, since paraffin sections are better for resolution of tissue architecture than frozen sections. This group of samples included 43 OSCCs, and 16 NCMTs that had well-oriented matched epithelial tissue components localized >1 cm away from corresponding tumour. IHC analysis was not carried out on the remaining 27 NCMTs due to limited tissue availability, vicinity to tumour <1 cm or disturbance of the epithelial orientation in the tissue section. Ninety-one per cent (39 cases) of these patients were areca chewers. The most common OSCC site was the buccal mucosa (63%, 27 cases) followed by the tongue (21%, 9 cases). With histopathological grading, 37% (16 cases), 56% (24 cases) and 7% (3 cases) showed well, moderate and poor differentiation, respectively; 46% (20 cases) of the patients had lymph-node metastasis and 46% (20 cases) had stage IV OSCC. Of the NCMTs, 44% (7 cases) exhibited focal or extensive epithelial hyperplasia or hyperkeratosis, and none exhibited epithelial dysplasia.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepared using a Tri-reagent® RNA isolation kit (Molecular Research Center, Cincinnati, OH, USA) and was treated with DNase I (Stratagen, La Jolla, CA, USA) to remove the contaminated DNA. The reverse transcription reaction was performed following protocols previously used. Sense primer (5′-ACAGGTTGGGATGGGATAGG-3′) and antisense primer (5′-ATATGAAGGAGGGGGTGAGG-3′) were used to generate a 203-bp *Pim-1* amplicon. Parallel amplification of *β-actin* in each reaction using primers (5′-ACACTGTGCCCATCTACGAGG-3′ and 5′-AGGGGCCGGACTCGTCATACT-3′) produced an amplicon of 621 bp as an internal control. The cycle number of the PCR was optimized by pilot studies to assure that the amplification was within the logarithmic phase. After electrophoresis, the densities of the amplicons were measured by a densitometer (Amersham, Piscataway, NJ, USA). The signal of *Pim-1* was normalized with *β-actin* to represent *Pim-1* mRNA expression. OSCCs exhibiting a *Pim-1*/*β-actin* ratio higher than that of the corresponding NCMT were defined as having increased *Pim-1* mRNA expression[4]. PCRs without template cDNA served as negative controls. Two individual RT-PCRs were performed for each case.

IHC

The IHC procedures followed protocols previously established. Anti-*Pim-1* monoclonal antibody (Santa Cruz Biotech., Santa Cruz, CA, USA) at 1:250 dilution was used as primary antibody. For the negative controls, the primary antibody was omitted. Small nests of greater than 100 cells accommodating the high-power field (×400) examination were evinced at random, and the total number of tumour cells was counted along with the number of immunopositive cells displaying distinctive immunoreactivity in accordance with the score method previously developed[3,4]. A total of 5 random fields containing greater than 500 tumour cells were examined for each case. Specimens containing <10% positive cells were defined as negative for immuoreactivity and specimens containing 10%–50% and ≥50% positive cells as weak (+) and strong (++) for immunoreactivity, respectively.

Statistics

Paired *t*-test, Fisher's exact test and Kaplan–Meier survival analysis were used for statistical analysis. The results ($P < 0.05$) were considered statistically significant.

Results

Pim-1 mRNA expression in tissue pairs

A representative analysis for *Pim-1* mRNA expression is shown in Fig. 1A. In 36 tissue pairs, *Pim-1* mRNA expression was 1.33 ± 0.41 for OSCCs and 0.97 ± 0.29 for NCMTs; this difference is statistically significant ($P = 0.03$, paired *t*-test) (Fig. 1B). Twenty-five (69%) OSCCs exhibited an increase in *Pim-1* mRNA expression (Fig. 1C). There was no correlation, however, between *Pim-1* mRNA expression and clinicopathological features including areca chewing, site, clinical stage, differentiation grade, lymph-node metastasis and patient age (detailed analysis not shown). There were 25 patients available for continuous follow-up of duration 22.9 ± 3.4 months; no significant association between *Pim-1* mRNA expression and survival was found (Fig. 5).

Pim-1 immunoreactivity in tissue pairs

IHC analysis of *Pim-1* was performed on sections of 43 OSCCs and 16 NCMTs. Cytoplasmic *Pim-1* immunoreactivity was seen in epithelial cells, inflammatory cells, endothelial cells and some fibroblasts (Fig. 2). In 16 NCMTs, 3 (19%) displayed strong immunoreactivity (++), 11 (69%) weak immunoreactivity (+) in the superficial half of the epithelium (Fig. 2A), and 2 (13%) no immunoreactivity (−) (Fig. 2C). *Pim-1* immunoreactivity was remarkably stronger in the OSCC counterparts (Fig. 2B and C): 26 (60%) OSCCs displayed strong *Pim-1* immunoreactivity (++) (Fig. 2B), 13 (30%) weak immunoreactivity (+) (Fig. 2C), and 4 (10%) no immunoreactivity. Within a specimen, the