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Peroxiredoxin-2 and zinc-alpha-2-glycoprotein as potentially combined novel salivary biomarkers for early detection of oral squamous cell carcinoma using proteomic approaches

Chukkris Heawchaiyaphum[a,f], Chamsai Pientong[a,f], Pensiri Phusingha[a,f], Patravoot Vatanasapt[b,f], Supannee Promthet[c,f], Jureerut Daduang[d,f], Watchareporn Teeramatwanich[b,f], Bunkerd Kongyingyoes[e], Jureeporn Chuerduangphui[a,f], Tipaya Ekalaksananan[a,f,*]

[a] Department of Microbiology, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand
[b] Department of Otorhinolaryngology, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand
[c] Department of Epidemiology, Faculty of Public Health, Khon Kaen University, Khon Kaen, Thailand
[d] Department of Clinical Chemistry, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, Thailand
[e] Department of Pharmacology, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand
[f] HPV & EBV and Carcinogenesis Research Group, Khon Kaen University, Khon Kaen, Thailand

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ABSTRACT

No effective screening method is available for oral squamous cell carcinoma (OSCC) that is recognized to influence by environmental factors as well as human papillomavirus (HPV) and Epstein-Barr virus (EBV). Therefore, we sought to identify salivary biomarkers for screening of OSCC with or without HPV and/or EBV infection. Saliva, lesion and oral exfoliated cells were collected from OSCC patients and cancer-free controls (CFCs) and grouped depending on their HPV- and EBV-infection status. Salivary protein was precipitated and subjected to 2-dimensional gel electrophoresis. Differential expression of proteins was identified by mass spectrometry and validated by Western blotting. Distinctive expression patterns of salivary proteins were detected in OSCC as compared with CFCs. Levels of peroxiredoxin-2 (PRDX-2) and zinc-alpha-2-glycoprotein (ZAG) were significantly up-regulated in OSCC cases ($p < 0.001$) relative to CFCs. Similarly, these proteins were also up-regulated in lesion cells compared with oral exfoliated cells ($p < 0.001$). However, the expression patterns of these proteins were not significantly influenced by patient histories (risk factors). In combination, these proteins yielded the highest discriminatory power (AUC = 0.999), sensitivity (100%), and specificity (98.77%) in distinguishing the early stages of OSCC. The detection of PRDX-2 combining with ZAG protein could potentially be used as salivary biomarkers for early screening of OSCC.

Significance: Our findings demonstrate a useful of combined detection of PRDX-2 and ZAG as a salivary biomarker for the early detection of OSCC.

1. Introduction

Oral squamous cell carcinoma (OSCC) that arises from the lining of the oral cavity is the eighth leading cause of cancer worldwide and is most prevalent in developing countries [1]. Oral carcinogenesis is clearly a multistep process influenced by risk factors including tobacco use, alcohol consumption, betel quid chewing and infection by tumor viruses. In the last category, human papillomavirus (HPV) and Epstein-Barr virus (EBV) are of particular importance and have been defined as risk factors for head and neck cancer (HNC) [2,3]. The clinical management of OSCC is dependent on several factors such as the early detection of the primary tumor and site-specific control of the disease [4,5]. OSCC patients are usually detected at an advanced stage, which results in a high mortality rate, poor prognosis and low 5-year survival rate (~50%) [1,6]. Therefore methods for the early detection of OSCC and screening methods for the at-risk population are urgently needed.

To date, the "gold standard" method for diagnosis of OSCC is histological analysis of a biopsy of the suspicious lesion. However, this method is invasive, expensive, and requires specially trained medical personnel. These limitations render it unsuitable for the early detection

* Corresponding author at: Department of Microbiology, Faculty of Medicine and HPV & EBV and Carcinogenesis Research Group, Khon Kaen University, Khon Kaen, Thailand.
E-mail addresses: chapie@kku.ac.th (C. Pientong), patvat@kku.ac.th (P. Vatanasapt), supannee@kku.ac.th (S. Promthet), jurpoo@kku.ac.th (J. Daduang), bunkon@kku.ac.th (B. Kongyingyoes), tipeka@kku.ac.th (T. Ekalaksananan).

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of OSCC and for mass screening [7,8]. A number of recent studies have explored tests based on saliva because this contains many proteins that may be associated with pathophysiological conditions, in particular systemic disease [9]. Compared to blood and biopsy samples, the use of saliva for screening tests for diseases is advantageous because sample collection and processing are easy and the procedure is minimally invasive, low cost and better tolerated by patients. In addition, saliva testing may be an effective strategy for screening, diagnosis, prognosis and monitoring post-therapy status [10]. Therefore, the analysis of the human saliva proteome may contribute to the understanding of pathophysiology and provide a foundation for the recognition of potential biomarkers of human diseases.

Previous studies have investigated saliva as a source of biomarkers for detection of OSCC. Using matrix-assisted laser desorption/ionization–mass spectrometry (MALDI-MS) and liquid chromatography with-tandem mass spectrometry (LC-MS/MS), Hu et al. identified thioredoxin as a candidate saliva biomarker for OSCC with a specificity and sensitivity of 70.8% [11]. Later, they identified a combination of five candidate biomarkers including mac-2 binding protein (M2BP), migration inhibitory factor-related protein 14 (MRP14), CD59, catalase, and profilin as salivary biomarkers for detection of OSCC. This combination yielded a high receiver operating characteristic (ROC) value, sensitivity (90%) and specificity (83%) [12]. The up-regulation of zinc finger protein 510 (ZNF510) peptide was also identified as a salivary biomarker for OSCC patients using proteomic approaches, with a high discriminatory power as indicated in AUC values (0.95 for early stage vs. control and 0.98 for advanced stage vs. control) [13].

Using ultra-performance liquid chromatography–mass spectrometry (UPLC–MS), Wang et al. identified a combination of four salivary metabolites (choline, betaine, pipecolinic acid and L-carnitine) that were useful as biomarkers for OSCC detection. This combination yielded a high sensitivity (100%) and specificity (96.7%), and satisfactory accuracy (0.997), at early stages of OSCC [14].

People in Northeast Thailand have unique dietary behaviors and different environmental factors to populations in many other regions of the world. Little research has been done on salivary protein biomarkers for OSCC detection in this region. Our previous studies have shown that the infection with HPV and EBV was significantly associated with OSCC, especially in patients who have the habit of chewing betel quid [15,16]. To date, there are no effective biomarkers for HPV- and EBV-associated OSCC, a disease which is increasing in the developing world. Therefore, this study aimed to investigate and identify potential salivary biomarkers for the early detection of OSCC influenced by environmental risk factors and also attempted to identify salivary biomarkers that might indicate whether OSCC is associated with HPV and/or EBV.

2. Materials and methods

2.1. Patients and study design

In this case-control study, ninety-two OSCC patients and eighty-three cancer-free controls (CFCs) were recruited. They were initially evaluated and/or diagnosed by an otorhinolaryngologist at the Department of Otorhinolaryngology, Srinagarind Hospital, Faculty of Medicine, Khon Kaen University. OSCC patients and CFCs were matched in term of gender and age. The inclusion and exclusion criteria for specimen collection from case and control were previous described by our colleagues [15,16]. The demographic data of all participants in this study are shown in Table 1. This study was conducted under the approval of Khon Kaen University Ethics Committee for Human Research (No. HE561407).

In this study, lesion cells and oral exfoliated cells were collected from sixty-three OSCC patients and sixty-four CFCs, respectively. Saliva samples were collected from ninety-two OSCC patients and eighty-three CFCs. Saliva samples were further separated into two sets, the discovery and the confirmation sample sets (Supplement Fig. S1). In addition, the saliva samples were grouped according to the HPV and EBV infection status of participants as follows: i) OSCC without viral infection, ii) OSCC and HPV infection, iii) OSCC and EBV infection and iv) OSCC and co-infection with HPV and EBV. The presence of HPV and EBV DNA in these samples was previously determined by our colleagues [15,16].

Table 1
Demographic data of CFCs and OSCC patients.

Demographic	Discovery set (n = 25)		Confirmation set (n = 150)	
	OSCC (n = 20)	CFC (n = 5)	OSCC (n = 72)	CFC (n = 78)
Age, year	60.35	47.50	63.51	64.53
Range	37–78	35–81	13–85	23–84
Sex				
Male	12	2	21	29
Female	8	3	52	49
Smoking history				
Yes	12	–	18	18
No	8	5	54	60
Alcohol history				
Yes	17	2	35	32
No	3	3	37	46
Betel quid chewing history				
Yes	5	1	44	18
No	15	4	28	60
Stage				
I/II	6		35	
II/IV	14		38	
Site of OSCC				
Tongue	11		28	
Lip	1		19	
Buccal mucosa	3		10	
Gum	2		11	
Floor of mouth	2		4	
Palate	1		–	

2.2. Biological specimen collection and protein extraction

Following collection, saliva samples (1 ml) were centrifuged at 12,000 rpm for 15 min at 4 °C. The supernatant was collected. Protease inhibitor mix (GE Healthcare, Piscataway, NJ) was added to give a final concentration of 1 × in the supernatant, and the mixture was aliquoted into 500 µl tubes for storage at − 80 °C. The protein precipitation method was performed as previously described by Jessie et al. [17]. The detail methods of this part were showed in Supplement Materials and Methods. In addition, the method for the collection of lesion cells and oral exfoliated cell was previously described by our colleagues [15,16].

2.3. 2-Dimensional gel electrophoresis (2-DE), in gel digestion and LC-MS/MS

Salivary proteins from OSCC patients and CFCs were pooled in groups of five according to their OSCC and viral infection status (Supplement Fig. S1). 2-DE was performed to profiling the expression patterns of saliva protein in OSCC patients and CFCs. The differential expression of proteins were identified by liquid chromatography with-tandem mass spectrometry (LC-MS/MS) and further validated by Western blotting. The detail methods of this part were showed in Supplement Materials and Methods.

2.4. Western blot analysis

Following separation of salivary proteins, by SDS-PAGE, 4 candidate proteins including haptoglobin (HP), Ig alpha-2 chain C region (IGHA2), peroxiredoxin-2 (PRDX-2) and zinc-alpha-2-glycoprotein