RESEARCH ARTICLE

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Comparison of DPPIV Levels in Serum and Tumour of OSCC Patients and Its Correlation with Active Matrix Metalloproteinases 2 and 9

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Abstract

Introduction: The important role of Dipeptidyl Peptidase IV (DPPIV) has been reported in tumour progression of several human cancers. This study demonstrates the DPPIV mRNA expression level and activity in tumour and paired non-tumour tissues of oral squamous cell carcinoma (OSCC) patients and the potential modulation of DPPIV in the metastasis of tumour through regulating MMP2 and MMP9 activities. **Materials and Methods:** This study was conducted on 16 OSCC patients. The mRNA expression level of DPPIV was evaluated by RT-qPCR in tumour of OSCC patientsand compared with their paired non-tumour tissues. Additionally, DPPIV activity was measured in serum, tumour and paired non-tumour tissues of OSCC patients. Zymography was performed to measure and compare the activities of MMP2 and MMP9 between tumour and paired non-tumour tissues of OSCC patients. **Results:** The results showed significantly higher DPPIV mRNA level and activity in tumour of OSCC patients compared to their paired non-tumour tissues. Tumour DPPIV mRNA expression and activity were positively correlated with activities of MMP2 and MMP9, respectively. Serum DPPIV activity of OSCC patients was lower compared to healthy control and did not show correlation with tumour DPPIV mRNA level. **Conclusion:** These data indicate that secreted DPPIV may not originate from the tumour tissue of OSCC patients. Furthermore, increased DPPIV gene expression and activity in tumour of OSCC patients might be involved in the ECM degradation and invasion of OSCC through regulation of MMP2 and MMP9 activities.

Keywords: Oral Squamous Cell Carcinoma- Dipeptidyl peptidase IV- MMP

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Introduction

Head and neck squamous cell carcinoma (HNSCC) originates from mucosal epithelial cells and is the sixth most common cancer in the world, with 450,000 deaths and 890,000 new cases reported in 2018 (Johnson et al., 2020; Kiani et al., 2020).

About half of HNSCC occurs in the oral cavity, of which OSCC is the most common form of oral cancer and has a high mortality rate and poor prognosis (Heroiu Cataloiu et al., 2013). OSCC arises in different sites of the oral cavity, including the floor of the mouth, the basal mucosa, the alveolar ridges, the retromolar trigone of the upper and lower gums, and the hard palate (Nazarian et al., 2019; Lin et al., 2021). Despite advances in cancer diagnosis and treatment, the overall 5-year survival rate for OSCC is the lowest among malignancies (Rashid et al., 2018; Sasahira and Kirita, 2018).

Most patients with OSCC are diagnosed in late stages III or IV, which significantly reduces the chance of survival (Tsai et al., 2021). The invasion and metastasis of cancer cells rely on the activation of different proteolytic systems which modulate degradation and reorganization of the extracellular matrix (ECM). Matrix metalloproteinases (MMPs) are key mediators of matrix degradation and tumour aggressiveness. MMP-2 and MMP-9 have gelatinase activity which cleave the basement membrane components, type IV collagen, elastin and laminin (Björklund and Koivunen, 2005). Upregulated expression and activity of fibroblast activation protein-α (FAP-α), DPPIV and MMps have been reported in esophageal cancer (Augoff et al., 2014).

DPPIV is one of a six-member family of serine proteases that have amino peptidase activity after proline (Chen and Kelly, 2003). This enzyme is a transmembrane homodimeric glycoprotein complex with a molecular weight of 200 kDa whose gene is located on chromosome 2 (Chen and Kelly, 2003; Augoff et al., 2014).

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DPPIV as a multifunctional enzyme is involved in multiple cellular processes including cell growth, migration, invasion, angiogenesis, and immune activation (Boonacker and Van Noorden, 2003). These various functions of DPPIV depend on its intracellular or extracellular localization, ligand concentrations, cell type and cofactor (Boonacker and Van Noorden, 2003). Proteolytic cleavage of type I and IV collagens by DPPIV and MMPs, respectively, alter the structure and mechanics of extracellular scaffolds and facilitates metastasis and cell migration (Augoff et al., 2014). Additionally, HNSCC and colorectal studies have shown that DPPIV increases the expression of MMPs and its high level associates with increased levels of MMP2, MMP9, MMP13 and MT1-MMP, leading to invasion, metastasis and poor prognosis (Augoff et al., 2014; Bishnoi et al., 2019). Taking into account the various functions of DPPIV, the opposite effects of DPPIV have been shown in different cancers. For instance, overexpression of DPPIV has been shown to suppress the invasive potential of ovarian cancer cells whereas increased expression of DPPIV has been reported in prostate and thyroid cancers (Kotani et al., 1991; Wilson et al., 2000a; Kajiyama et al., 2003; Vitório et al., 2020).

DPPIV inhibitors used by lung and colorectal cancer patients were associated with improvement of their overall survival, the mechanism underlying this improvement could be through its effect on cancer immunoregulation (Bishnoi et al., 2019). However, other studies have shown that the use of DPPIV increase increase increase the risk of metastasis in the cell lines of the colon, liver, lung, ovary and melanoma (Bishnoi et al., 2019).

Considering the importance of DPPIV in cancer biology and its possible correlation with clinicopathological features of OSCC patients, we report DPPIV mRNA expression and activity in tumour and paired non-tumour tissues as well as DPPIV activity in the serum of OSCC patients. We also evaluate whether the serum DPPIV could originate from tumour tissue of OSCC patients. In addition, we investigate the correlation between DPPIV gene expression/activity and MMP2 and MMP9 activity.

Materials and Methods

Subjects and study design

A total of 32 tissue (16 tumour and 16 paired non-tumour tissues) and serum from OSCC (n = 24) and healthy individuals (n = 16) were collected for this study, OSCC patients who went under surgery at Bahman and Shariati hospital of Tehran, Iran. Healthy subjects of control group for serum study were age- and gender-matched without any history of major illness in the past. Blood samples were obtained from all the participants before surgery. Blood was collected into sterile BD Vacutainer tubes. Next, the tubes were centrifuged at 3000 rpm for 10 min and serum was separated immediately and stored at -70°C until use for DPPIV activity analysis.

All of the tumours were diagnosed as grade I to III according to the WHO criteria. Subjects with diabetes, liver and autoimmune diseases were excluded. Informed consent from all patients for the analysis of their tissues was collected before surgery. This study was approved by the ethics committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.MSP.REC.1400.607). Table 1 represents the methods used for the evaluation of DPPIV in serum and tissue samples of OSCC patients.

Zymography

The extraction of total protein from frozen tissues was performed by homogenization 1:10 w/v in RIPA buffer supplemented with PMSF, sodium orthovanadate and protease inhibitors. Next, the homogenates were centrifuged for 15min at 12000 rpm at 4^{o}C and the supernatant was collected. The protein concentration was determined with the Bradford method. For determining of gelatinolytic activity of the MMP2 and MMP9, substrate gel SDS-PAGE zymography was performed according to (Toth et al., 2012) with the following modifications: A total of 40 µg protein of each sample with none-reducing sample buffer 4x containing 0.25 M Tris-HCl (pH = 6.8) with 40% glycerol, 8% SDS and 0.01% bromophenol blue, was loaded in 10% SDS-polyacrylamide gel copolymerized with gelatin 0.1%. After electrophoresis, the enzymes were renatured by washing SDS out twice with a renaturing solution containing 2.5% Triton X-100 for 30 min at room temperature (RT). Next, the gel was incubated in developing buffer containing 50 mM Tris-HCl (pH = 7.5), 200 mM NaCl, 5 mM $CaCl_2$, 1 µM $ZnCl_2$ and 0.02% Brij-35 for 30 min at RT which was followed by another incubation in developing buffer at 37˚C overnight. To visualize the proteolytic bands, 5% v/v methanol, 10% v/v acetic acid, and 0.5% Coomassie blue R-250 were used to stain the gel, which was then destained with 10% acetic acid and 5% methanol. Densitometric measurements were conducted using an ImageJ gel analyzer to record the intensity of the pixels (inverted) of the bands.

RNA isolation and real-time quantitative q-PCR (RT-qPCR)

Total RNA was isolated from tumour and paired non-tumour tissues of OSCC patients using Trizol reagent (GENE All) and cDNA was synthetized with a First Strand cDNA Synthesis Kit (Takara, Japan) according to the manufacturer's protocol. RT-qPCR was performed using Ampliqon master mix on StepOne Plus RT-PCR system. GAPDH was used as internal control. Each sample was performed duplicate and the data was normalized to GAPDH. Expression levels have been determined using $2^{-\Delta\Delta Ct}$ method. The sequences of primers were as follows: DPPIV forward: 5'-AGTGGCGTGTTCAAGTGTGG-3' and DPPIV reverse: 5'-CAAGGTTGTCTTCTGGAGTTGG-3' and GAPDH forward: 5'-GCTCAGACACCATGGGGAAG-3' and GAPDH reverse 5'-TGTAGTTGAGGTCAATGAAGGGG-3'.

DPPIV activity in tumour, non-tumour and serum of OSCC patients

DPPIV activity was measured colorimetrically using glycyl-prolyl-paranitroanilide (Gly-Pro-pNA) substrate based on method described in (Matheeussen et al., 2012). Briefly, 0.5 ml of 100 mM Tris-HCl (pH = 8) was added to 30-50 mg of tumour and non-tumour tissues and incubated

on ice, then homogenates were centrifuged for 12min at 12,000 rpm at 4°C. After collection of the supernatant, 0.5 ml of 100 mM Tris-HCl (pH = 8) containing 2% Triton X-100 (Pierce) was added to the pellet and vortexed. Suspension was centrifuged for 12 min at 12,000 rpm, at 4°C.

10 µl of serum and supernatants recovered from tissue samples were incubated with substrate Gly-Pro-pNA 0.5 mM, in 50 mM Tris buffer (pH = 8.3) and the kinetic activity of DPPIV was assessed immediately by measuring the velocity of pNA release (405 nm) from the chromogenic substrate for 15min at 37°C. Based on the total protein content of the tissue, DPPIV activity was measured and expressed as nanomoles of substrate converted per milligram protein of tissue per minute.

Statistical analysis

The data was analyzed using GraphPad Prism version 6 and presented as an average standard deviation (SD). Data normality was evaluated by the Shapiro–Wilk test. Statistical analysis was done using the paired student's t-test. The correlation coefficients were calculated based on Spearman's correlation two tailed analysis. P-values <0.05 were considered statistically significant in all cases.

Results

Tissue DPPIV mRNA expression and activity

To determine if the tumour and paired non-tumour tissues of OSCC patients could present difference in DPPIV gene expression, we performed RT-qPCR. As observed, tumour tissues presented higher DPPIV mRNA level (p = 0.0056) and activity (p = 0.0013) compared to their paired non-tumour tissues (Figures 1A and B). Next, we questioned if DPPIV mRNA level correlates with the enzyme activity, the results did not reveal correlation between expression level of DPPIV mRNA and its activity in tumour tissue of OSCC patients (Table 2).

Table 1. Methods Used for the Study of Proteolytic Enzymes.

Sample	RT-qPCR	DPPIV activity	MMP2 and MMP9 (Gelatin zymography)
Tumour and paired non-tumour tissues	Yes	Yes	Yes
Serum		Yes	

DPPIV, dipeptidyl peptidase IV; MMP, matrix metalloproteinase

Serum DPPIV activity

The serum DPPIV activity was significantly lower in OSCC patients compared to the normal group (p = 0.0354) (Fig. 1C). Next, we questioned if the higher tumour DPPIV mRNA and activity correlate with the serum DPPIV, in another words if the serum DPPIV could originate from OSCC tumour, the results obtained were against this correlation (r = 0.025, p = 0.943) (Table 2).

Tissue enzyme activity of MMP2 and MMP9

The zymography data obtained from tumour and paired non-tumour of 16 OSCC patients showed higher active MMP2 (p = 0.0067) and MMP9 (p = 0.0002) in OSCC

Table 2. The Correlation between Tumour DPPIV Gene Expression and Activity with the Serum DPPIV Activity and the Levels of Active-MMP2 and Active-MMP9.

	Serum DPPIV activity		Tissue MMP-9 activity		Tissue MMP-2 activity	
	Spearman r	P-value	Spearman r	P-value	Spearman r	P-value
Tissue DPPIV activity	-0.053	0.848	0.448	**0.01**	0.436	**0.013**
Tissue DPPIV mRNA expression	0.025	0.943	0.118	0.663	0.504	**0.049**

Bold values represent significant correlations as evaluated by Spearman's correlation analysis.

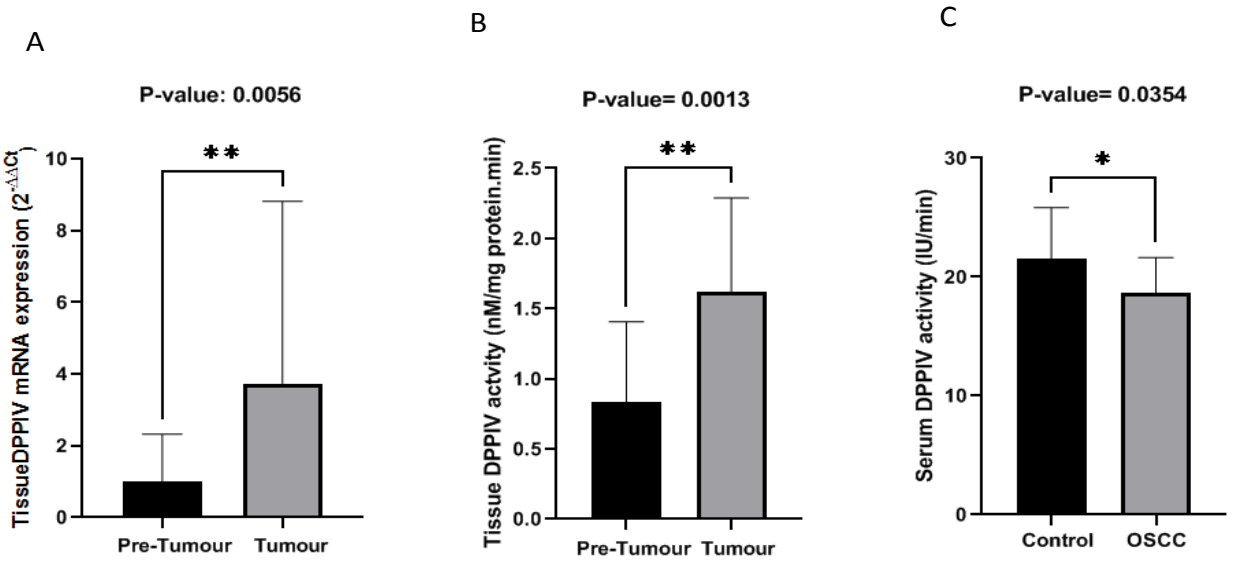

Figure 1. Expression and Activity of DPPIV in Tumour and Paired non-Tumour Tissues (A and B) and Serum of OSCC Patients (C) (n = 16). Values represents mean ± standard deviation (SD).

Table 3. The Correlation between DPPIV Gene Expression, Activity and the Levels of Active-MMP2 and Active-MMP9 with Clinical Features of OSCC Patients.

	Grade		Tumour site		Size		Sex		Age	
	Spearman r	P-value	Spearman r	P-value	Spearman r	P-value	Spearman r	P-value	Spearman r	P-value
Serum DPPIV activity	0.168	0.567	0.237	0.265	0.281	0.259	0.65	**0.001**	0.023	0.916
Tissue DPPIV activity	0.216	0.667	0.023	0.934	0	1	0.26	0.351	-0.304	0.251
Tissue DPPIV mRNA expression	-0.378	0.25	0.093	0.731	0.426	0.147	-0.298	0.279	-0.046	0.867
Tissue MMP-9 activity	-0.309	0.567	-0.306	0.245	-0.064	0.854	-0.315	0.242	-0.515	**0.043**
Tissue MMP-2 activity	0.031	0.967	-0.154	0.564	-0.114	0.735	0.301	0.262	0.204	0.446

Bold values represent significant correlations as evaluated by Spearman's correlation analysis.

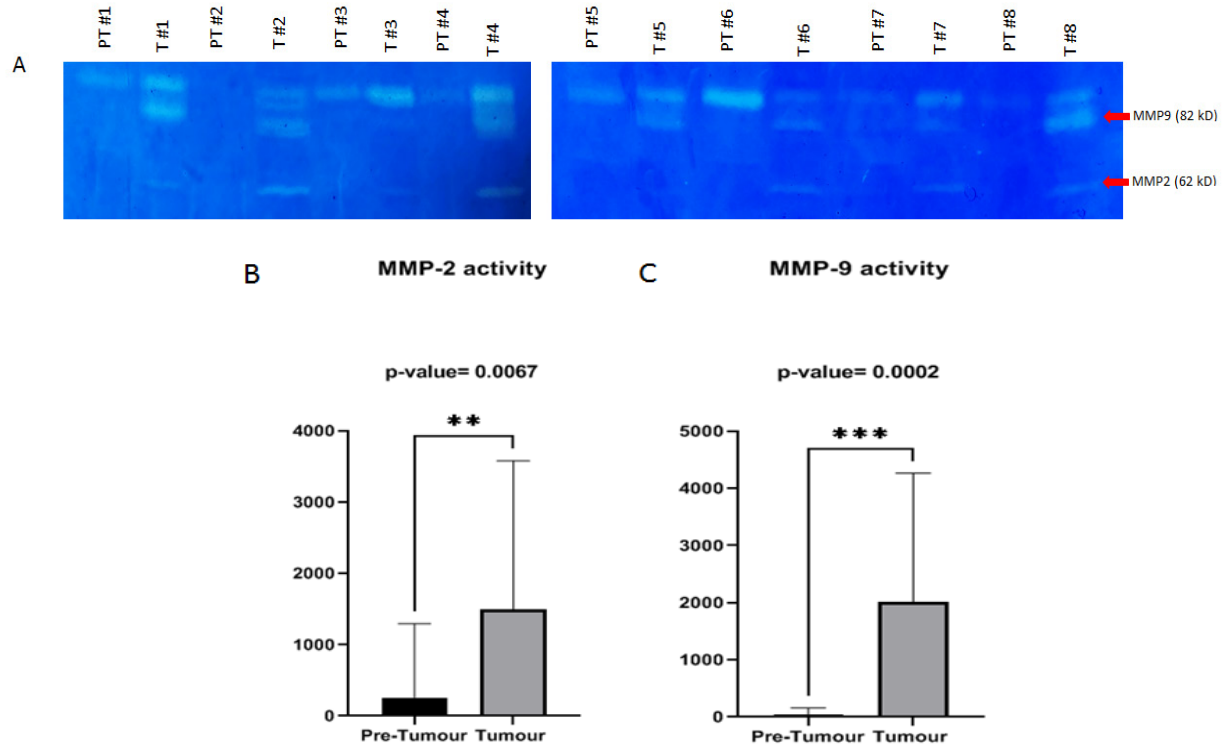

Figure 2. Gelatinolytic Activity in Tumour and Paired Non-Tumour Tissues of OSCC Patients. The zymography gel showing pro- and active forms of MMP2 (lower bands) and MMP9 (upper bands) in 32 paired tumour and non-tumour tissues (n = 16 OSCC patients) (A). Semi-quantitative analysis of active MMP2 and active MMP9 levels in the tumour and paired non-tumour tissues (B and C) (n = 16), Zymograph of 8 OSCC patients is shown. PT and T represent peri-tumour and tumour tissues, respectively. Values represents mean ± standard deviation (SD).

tumour tissues compared with their paired non-tumour tissues (Figures 2B and C).

Correlation between tissue DPPIV mRNA level and activity of active MMP-2 and MMP-9

In this study, we have measured the tumour DPPIV mRNA level and activity and also analyzed whether DPPIV gene expression and activity is associated with the active form of MMP-2 and MMP-9. Table 2 shows that DPPIV activity in tumour tissues correlates positively with active-MMP-2 (r = 0.436, p = 0.013) and active MMP-9 activity (r = 0.448, p = 0.01). Additionally, tumour DPPIV mRNA expression was correlated only with active-MMP2 activities (r = 0.504, p = 0.049).

Correlation between DPPIV mRNA level, DPPIV activity, MMP2 and MMP9 with clinical features of OSCC patients

Bivariate correlation analysis of DPPIV gene expression, DPPIV activity and the levels of active-MMP2 and active-MMP9 with OSCC patients' clinical features including age, gender, tumour size, grade and tumour location is shown in Table 3. There was no significant association between DPPIV mRNA level, DPPIV activity, active MMP2 and active MMP9 with grade, tumour size and tumour anatomical location. Serum DPPIV activity and active-MMP9 were significantly correlated with sex (r = 0.65, p = 0.001) and age (r = -0.515, p = 0.043), respectively. OSCC male patients had higher serum DPPIV activity than females (Table 3).

Discussion

OSCC is an aggressive, locally dominant tumour which in recent decades has become a major concern for global public health. Although the diagnosis and treatment

techniques of OSCC have improved in the past decades (Luo et al., 2020) but five years-survival rate is still lower than 50%. In order to have a better chance of survival, OSCC patients must be diagnosed and treated in the early stages (Luo et al., 2020). Therefore understanding the molecular mechanisms of OSCC to identify new therapeutic targets and developing reliable prognostic histological markers to predict biological behavior and improve stratification and management of OSCC patients is necessary (Ding et al., 2022; Li et al., 2022).

It has been proved that different tumour markers, including lipids, enzymes, polyamines, immunoglobulins, viral markers, glycoproteins, circulating immune complexes (CIC), hormones, tumour-associated antigens, and oncofetal proteins have been useful in studying human cancers (Mishra et al., 2021). A cell surface glycoprotein known as DPPIV plays multiple roles, including the regulation of glucose metabolism, immunomodulation, and tumour progression (Pan et al., 2021). Deregulation of DPPIV has been shown in various cancers, DPPIV overexpression is reported in thyroid cancer, ovarian cancer, prostate cancer, colorectal cancer, osteosarcoma and malignant mesothelioma (Kotani et al., 1991b; Wilson et al., 2000b; Kajiyama et al., 2003b; Inamoto et al., 2007; Lu et al., 2013; Zhang et al., 2013). In contrast, DPPIV downregulation is observed in melanoma (Havre et al., 2008). DPPIV has been extensively investigated as a cancer biomarker and a therapeutic target (Ohnuma et al., 2018). Taking into account diverse biological functions of DPPIV, the mechanism through which DPPIV affects tumour cell behavior may be different depending on the type of cancer, possibly resulting in a divergent effect. Recent studies have demonstrated that DPPIV overexpression suppressed invasive potential in ovarian cancer cells through downregulation of MMPs, as well as upregulation of tissue inhibitors of metalloproteases (TIMPs) (Kajiyama et al., 2003a; Kikkawa et al., 2005). According to other studies, loss of DPPIV expression is associated with tumour progression and malignant transformation (Morrison et al., 1993; Pro and Dang, 2004). In contrast, prostate and thyroid malignancies were found to exhibit enhanced DPPIV expression and DPPIV knockdown decreased proliferation, migration and invasion in urothelial carcinoma cell lines (Kotani et al., 1991b; Wilson et al., 2000a).

Considering the reported secretion of DPPIV from adipocytes and its contribution to the circulating DPPIV (Sell et al., 2013), we hypothesized on the possible secretion of DPPIV from tumour tissue of OSCC patients. Therefore, this research aimed to compare tissue DPPIV gene expression and activity with paired non-tumour tissues of OSCC patients as well as serum DPPIV enzyme activity between OSCC patients and healthy individuals and investigating the correlation between tissue DPPIV mRNA/ activity and serum DPPIV activity. Results showed that serum DPPIV is lower in OSCC samples compared with normal ones which is in agreement with findings reported by Fukasawa et al (Fukasawa et al., 1982; Urade et al., 1989), and Mishra et al (Mishra et al., 2021). The evaluation of tissue DPPIV mRNA expression and activity in tumour samples and their paired non-tumour samples showed higher tissue DPPIV mRNA expression and activity in tumour samples. In agreement to our findings Ding et al., (2019), and Augoff et al., (2014) demonstrated that the expression and activities of DPPIV in OSCC tissue were significantly higher than in their paired non-tumour samples. Furthermore, neither tissue DPPIV mRNA level nor tissue DPPIV activity showed correlation with their paired serum DPPIV activity which may indicate circulating DPPIV does not originate from tumour tissue of OSCC patients, considering low number of patients included in this study, additional researches with higher number of patients are needed to conclude whether OSCC tumour tissues secrete DPPIV or not. The results of the present study showed no significant correlation between serum DPPIV activity and age. This is in contrast with previous reported findings showing lower DPPIV activity in older adults (Neidert et al., 2016), which could be due to small sample size of our study. We also found higher serum DPPIV activity in men than women which is in contrast with the findings of Sanz et al., (2018). MMPs play a crucial role in cell migration, invasion and distant metastases by degrading the ECM (Kessenbrock et al., 2010). MMP-2 and MMP-9 are two members of the MMP family which are expressed in OSCC, and their expression has been shown to be significantly correlated with the invasion of OSCC (Lee et al., 2008; Yamada et al., 2016). Zymography of tumour samples and paired non-tumour tissues of OSCC patients showed that active MMP-2 and active MMP-9 were significantly higher in tumour samples. Published data have shown that DPPIV participates in the regulation of MMPs in ovarian carcinoma cells (Kajiyama et al., 2003a). Results of research conducted by Dings et al (Ding et al., 2019) on the assessment of DPPIV effects on the expression of E-cadherin, MMP2 and MMP9 in OSCC cell lines showed that high metastatic cell lines express significantly higher levels of DPPIV than non-metastasized ones, and siRNA suppression of endogenous DPPIV expression significantly reduced cell motility and invasion in highly metastatic cell lines through down regulation of MMP-9 expression. Correlating tissue DPPIV mRNA level and activity with the active MMP-2 and active MMP-9 levels, we observed that active MMP-2 and active MMP-9 had positive correlation with tissue DPPIV activity whereas only active MMP-2 showed positive correlation with tissue DPPIV mRNA level. The positive correlation between tumour DPPIV, MMP-2 and MMP-9 activities is in consistent with the findings of Ding et al on OSCC cell lines (Kajiyama et al., 2003; Ding et al., 2019).

In conclusion, we found higher DPPIV gene expression, enzymatic activity, active MMP-2 and active MMP-9 in tumour tissue of OSCC patients compared to their paired non-tumour tissues. Additionally, we observed lower DPPIV enzymatic activity in serum of OSCC patients compared to healthy individuals. No correlation was found between DPPIV mRNA level and enzymatic activity with the serum DPPIV activity. We found a positive correlation of tumour DPPIV activity with the levels of active MMP-2 and active MMP-9.

Here we report the possible correlation between the DPPIV enzymatic activity and active gelatinase

including MMP-2 and MMP-9. These findings suggest that coordinate function of DPPIV, MMP-2 and MMP9 may contribute to regulation of OSCC's behavior.

Author Contribution Statement

AG designed and supervised the study. ATT performed RT-qPCR, zymography and enzyme activity experiment under supervision of TL, MSS and AG. SK helped with enzyme activity experiment. AK provided patient's samples. ATT and AG performed statistical analyses and wrote the manuscript. All authors critically reviewed and approved the final version of the manuscript

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Availability of data

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Conflict of interest

The authors declare no competing interest.

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