EGYPTIAN DENTAL JOURNAL

VOL. 64, 1187:1202, APRIL, 2018

I.S.S.N 0070-9484

ORAL MEDICINE, X-RAY, ORAL BIOLOGY AND ORAL PATHOLOGY

www.eda-egypt.org • Codex : 79/1804

PROGNOSTIC SIGNIFICANCE OF FASCIN AND WILM'S TUMOR 1 (WT1) IN ORAL SQUAMOUS CELL CARCINOMAS – CASE CONTROL STUDY

Samah H. El-Meadawy*, Naglaa M. Salama**, Lobna R.S. Radwan*** *and* Mahmoud El-sherbiny****

ABSTRACT

Aims: To elucidate the role of fascin and WT1 expression in oral squamous cell carcinoma (OSCC) by correlation with clinicopathological parameters.

Subjects and methods: Paraffin sections of 27 OSCC tissue were immunohistochemically stained with fascin and WT1 using the avidin-biotin-peroxidase staining method. Correlations between fascin and WT1 and various clinicopathological features, and prognosis were studied.

Results: Immunohistochemical study revealed significant increase of fascin and WT1 in OSCC in relation to control group ($p< 0.000$). Receiver operating characteristic (ROC) curve for fascin and WT1 were conducted for detection of recurrence. Fascin showed fair area under curve (AUC) (AUC=0.66), with sensitivity of 63.6 % and specificity of 50.0 % at cutoff value of 42.5 %. WT1 also showed fair AUC (AUC=0.63), with sensitivity of 63.6 % and specificity 68.8 % at cutoff value of 40.0 %. ROC curve for WT1 and fascin were conducted for detection of lymph node infiltration. While fascin showed excellent AUC (AUC=0.865), cutoff value of 52.5 %, with sensitivity of 70.0 % and specificity of 94.1%. WT1 also showed excellent AUC (AUC=0.791), cutoff value of 42.2 %, with sensitivity of 70.0 % and specificity 70.6%. Significant associations were detected between median fascin cutoff value at 45% and well differentiated tumor (P=0.04), T3&T4 tumor size (P= 0.01), LN infiltration (P=0.05), and tumor-node metastasis 3 and 4 (TNM3 &TNM4) staging (P=0.02). Significant associations were found between the median cutoff value of WT1 and moderately differentiated tumor (P=0.02), and LN infiltration (P=0.01). The 2-year survival rate of patients with fascin of ≥ 45% and WT1 ≥ 40% were nonsignificantly higher than that of patients with fascin of < 45% and WT1 <40% (P=0.09, P=0.55). Univariate analysis demonstrated that Fascin, WT1 were significant risk factors of LN (p=0.005, 0.02) but not considered as risk factors of tumor recurrence.

Conclusion: Fascin and WT1 have oncogenic effects playing an important role in progression of OSCC. Overexpression of them contributes to a more aggressive clinical course. Understanding their role on OSCC and other tumors will facilitate the development of new treatment strategies.

KEY WORDS: Oral squamous cell carcinoma, fascin, Wilm's tumor 1 (WT1), tumor markers.

*Associate Professor of Oral Medicine and Periodontology, Department of Oral Medicine, Periodontology, Oral Diagnosis , and Radiology, Faculty of Dentistry, Mansoura University.

** Associate Professor of Oral Pathology, Oral Pathology Department, Faculty of Dentistry, Mansoura University.

***Associate Professor of Oral Biology, Oral Biology Department, Faculty of Dentistry, Mansoura University.

****Assistant Professor of Oral Pathology, Oral Pathology Department, Faculty of Dentistry, Mansoura University.

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the sixth most common malignancy in the world. It is a major cause of cancer morbidity and mortality [1]. Despite substantial developments in therapeutic strategies during the past decades, only 60% of affected individuals survive for 5 years [2]. Local recurrence and regional lymph node metastasis are two major hurdles in the management of the advanced stage OSCC [3]. Thus, a comprehensive investigation of the factors and molecular events which contribute to local recurrence and the invasion of OSCC are necessary for the development of novel strategies for prognostication and treatment.

Tumor invasion and metastasis are closely related to the cell transformation, loss of cell-cell adhesion, degradation of ECM and cell migration [4], resulting from rearrangements of the cytoskeletal microfilaments. Reorganization of the actin cytoskeleton is regulated by the action of actin cross-linking proteins [5].

Fascin is a 55-kD actin bundling protein. It has 2 major binding sites for actin, to allow efficient F-actin bundling in filopodia, lamellipodial ribs, dendrites, spikes, and microvilli [6]. Fascin is predominantly expressed in cells, which form membrane protrusions and require motility, such as neurons, glial cells and dendritic cells [7] and also in vascular endothelial cells and fibroblasts [8].

Fascin is also found to be involved in the formation of invadopodia and appears to aid tumor cell invasion [9]. Fascin regulates actin polymerization and cell motility in K8-knockdown OSCC cells. Decrease in fascin levels was also associated with reduced invasive ability and tumorigenicity in K8- depleted cells [10]. Although several studies have shown that fascin upregulation is associated with more aggressive and metastatic phenotypes in epithelial cancers [11-13], few studies have detected its promoting role in oral squamous cell carcinoma [14, 15].

Wilms' tumor 1 (WT1), a multifunctional transcription factor, is important for embryonic kidney, genital organs, heart, central nervous system, and blood development. WT1 regulates multiple cellular processes including apoptosis, proliferation, differentiation, and mRNA processing [16]. WT1 also affects cell division process by regulating the spindle/mitotic checkpoint function [17].

During embryonic life, wild type WT1 acts as a tumor suppressor and its mutation leads to Wilms' tumor of the kidney. While in adults, WT1 has been reported with diversing roles of a tumor suppressor and an oncogene. WT1 mutation [18] and overexpression of the WT1 gene or the WT1 protein were found to be related to carcinogenesis [19-20].

Up to our knowledge, only a few studies that detect WT1 protein expression in oral squamous cell carcinoma [19,21]. Therefore, the exact role of WT1 in OSCC tumorigenesis still is unclear. Thus, the aim of this study is to elucidate the role of fascin and WT1 in OSCC by correlation with clinicopathological parameters and to calculate a suitable fascin and WT1 cutoff value for predicting patient prognosis.

SUBJECTS AND METHODS

Twenty seven paraffin embedded blocks of primary OSCC tissues (study group) and the clinicopathological records of the patients were collected and retrieved from the Mansoura Oncology Centre, Egypt from July 2013 to June 2015. All 27 study cases were clinically staged according to the tumor-node metastasis (TNM) system [22]. Thirty normal oral mucosal tissue samples were taken as a reference (control group) from healthy subjects during extraction of impacted third molars. This study was approved by the institutional review board of the Faculty of Dentistry, Mansoura University. Informed consents were obtained from the patients as well as healthy individuals for inclusion in the study.

Immunohistochemistry (IHC):

Paraffin sections (5-μm thick) of OSCC tissue were immunohistochemical stained with fascin and WT1 using the avidin-biotin-peroxidase staining method. Immunohistochemical analysis of formalin fixed, paraffin embedded specimens was performed according to the manufacturer's instructions [23]. Briefly, after deparaffinization and rehydration, heat-induced antigen retrieval was performed, the tissue sections were treated with 0.1 M sodium citrate (pH 6.2). Endogenous peroxidases were blocked with 0.9% hydrogen peroxide.

Then, the slides were preincubated with 10% normal serum in 2% bovine serum albumin (BSA) / phosphate buffered saline (PBS) for 20 min (to avoid unspecific binding). Sections were incubated with the following primary antibodies: mouse monoclonal antibody (1:50; Dako Deutschland GmbH, Hamburg, Germany) overnight at 4°C and mouse anti-human WT1 monoclonal antibody (1:100, M3561; Dako) at room temperature for 1 h. The slides were incubated with a biotinylated secondary antibody (1:100; DAKO Deutschland GmbH), streptavidin peroxidase (1:100; Dianova GmbH, Hamburg, Germany) and 3,3'-diaminobenzidine/ H_2O_2 (1.85 mM). The slides were counter stained with hematoxylin solution.

The staining reaction was assessed using Bittinger et al scoring system [24]. Immunostainning of each slide were assessed for fascin and WT1 as percentage of positive stained tumor cells (number of positive stained cells divided by the total number of examined cells in five selected fields) of the highest proliferative activity at ×400 magnification. Immunohistochemical staining were assessed independently by two observers and the mean of their readings were taken.

Statistical analysis:

Data were entered and statistically analyzed using the Statistical Package for Social Sciences (SPSS) version 20.

Qualitative data were described as numbers and percentages with Chi-Square test used for comparison and Fischer exact test was used when more than 20% of cells have counted less than 5.

Quantitative data were described as median and range after testing normality by Kolmogorov-Smirnov test. Kruskal-Wallis test and Mann-Whitney tests were used for comparison between groups. Receiver operating characteristic (ROC) was used to calculate the validity (sensitivity & specificity) of continuous variables with calculation of the best cutoff point. Kaplan Meier curve was used for calculation of median survival time and comparing the marker effect on survival with log rank test calculation, the median value of fascin & WT were used as an arbitrary cutoff point. Binary stepwise logistic regression analysis was used for prediction of independent variables of lymph node infiltration and recurrence. Significant predictors in the bivariate analysis were entered into the regression model using forward Wald method. Adjusted odds ratios and their 95% confidence interval were calculated.

p value ≤ 0.05 was considered to be statistically significant. All tests were 2-tailed.

RESULTS

In this study, the OSCC group (27 subjects) included 20 male and 7 female patients with 11 patients with an age $\geq$ 60 (age range: 60-75 years) and 16 patients with an age < 60 years (age range 48-59 years). These tissue samples included 12 SCC of the tongue, 6 SCC of buccal mucosa, 3 SCC of gingiva, 3 SCC of lips, 2 SCC of palate, 1 SCC of retromolar area. According to T-stage: T1 includes 4 cases, T2 includes 9 cases, T3 includes 10 cases, and T4 includes 4 cases. Lymph node infiltration was found only in 10 patients. All patients showed no distant metastasis and recurrence occurs in 11 cases only. The patients showed different TNM grading: TNM1 (4 cases), TNM2 (7cases), TNM3 (9 cases),

and TNM 4 (7 cases). The tissue samples included 10 well differentiated OSCC, 10 moderately differentiated OSCC and 7 poorly differentiated OSCC. The control group (30 subjects) were 12 males and 18 females with an age range 25-35 years.

Immunohistochemical results show a significant increase in the expression of fascin (figure 5) and WT1 (figure 6) in the cytoplasm of tumor cells than that in control biopsies. It was found that the median of fascin in OSCC biopsies was 45% (17%-85%) and 0.000 (0-5%) in control with significant differences (Z=6.378, p< 0.000). The median of WT1 in SCC biopsies 40% (15%-80%) was significantly higher than that in control biopsies 3 (2-4) with Z=6.374, p< 0.000.

Table 1 also shows median fascin & WT1 score distribution according to clinicopathological features of oral squamous cell carcinoma patients. No significant differences in fascin and WT1 distribution were found as regard to age, sex, site, and recurrence. There were significant differences in fascin distribution between poorly differentiated

Figure 1: AUC and performance characteristics of WT1 and fascin for detection of lymph node infiltration:

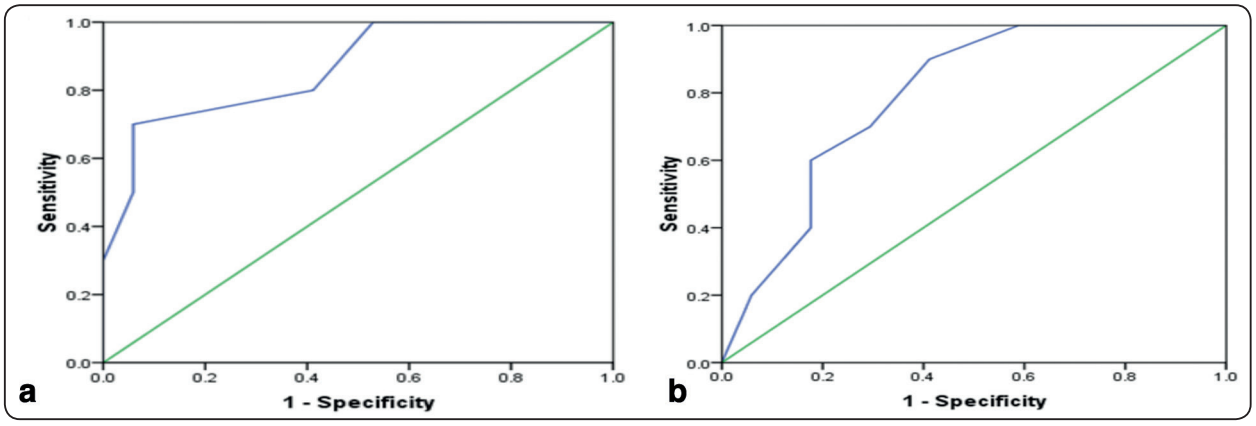

Fig. (1): (a)ROC curve showing validity of fascin in detection of lymph node infiltration of squamous cell carcinoma. (b): ROC curve showing the validity of WT1 in the detection of lymph node infiltration of squamous cell carcinoma

Figure 2: AUC and performance characteristics of WT1 and fascin for detection of recurrence:

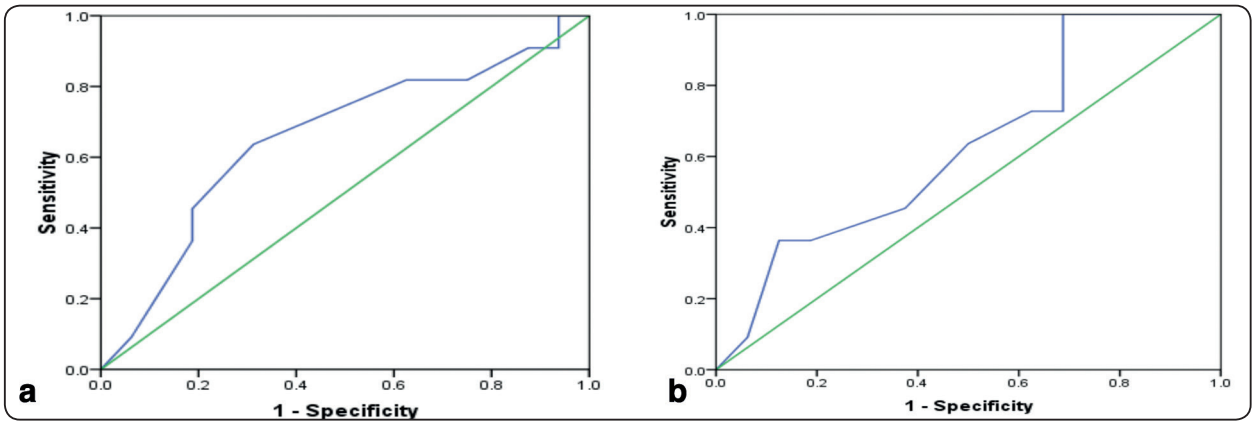

Fig. (2): (a)ROC curve showing validity of fascin in detection of recurrent Squamous cell carcinoma. (b): ROC curve showing validity of WT1 in detection of recurrent Squamous cell carcinoma

OSCC and both of well differentiated and moderately differentiated OSCC (P=0.003). There was also a significant difference between WT1 of poorly differentiated and well differentiated OSCC (P= 0.003). Fascin distribution of OSCC of T3 &T4 was significantly higher than that of OSCC of T1 &T2 (P=0.004). Fascin and WT1 distribution were significantly higher in OSCC associated with lymph node infiltration than OSCC without LN infiltration (p=0.002, 0.012). Moreover, OSCC with TNM3&4 showed significant fascin and WT1 distribution than OSCC with TNM1&2 (P= 0.001, =0.03)

Table 2 shows associations between the clinicopathological features of oral squamous cell carcinoma and median cutoff value of fascin. We divided the patients into two groups using a fascin

TABLE (1) Median fascin & WT1 score distribution according to clinicopathological features of oral squamous cell carcinoma patients:

	N	%	fascin	P value	WT1	P value
Age						
• <60	16	59.3	45.0 (20.0-85.0)	Z=1.2	40.0(20.0-80.0)	Z=0.79
• ≥60	11	40.7	24.0(17.0-80.0)	P=0.23	35.0(15.0-80.0)	P=0.43
Sex						
• Male	20	74.1	45.0(17.0-85.0)	Z=1.15	42.5(15.0-80.0)	Z=0.72
• Female	7	25.9	30.0(17.0-85.0)	P=0.25	35.0(15.0-70.0)	P=0.47
Site						
• Buccal	6	22.2	32.5(17.0-45.0)	KW	37.5(20.0-70.0)	KW
• Gingival	3	11.1	45.0(45.0-70.0)	P=0.21	75.0(20.0-75.0)	P=0.21
• Lip	3	11.1	60.0(30.0-80.0)		40.0(35.0-45.0)	
• Palate	2	7.4	20.0(20.0-20.0)		27.5(15.0-40.0)	
• Retro	1	3.7	70.0(70.0-70.0)		70.0(70.0-70.0)	
• Tongue	12	44.4	45.0(17.0-85.0)		42.5(15.0-80.0)	
Grade						
• Poor	7	25.9	70.0^{AB} (30.0-85.0)	KW	70.0^{A} (35.0-80.0)	KW
• Moderate	10	37.0	45.0^{B} (20.0-60.0)	P=0.003**	42.5 (20.0-75.0)	P=0.003**
• Well	10	37.0	20.0^{A} (17.0-70.0)		27.5^{A} (15.0-80.0)	
Tumor size						
• T1&T2	13	48.1	24.0(17.0-70.0)	Z=2.9	35.0(15.0-80.0)	Z=1.66
• T3&T4	14	51.8	52.5(20.0-85.0)	P=0.004**	45.0(20.0-80.0)	P=0.09
LN involved						
• N0	17	63.0	30.0(17.0-70.0)	Z=3.15	35.0(15.0-80.0)	Z=2.5
• N1	10	37.0	67.5(30.0-85.0)	P=0.002**	62.5(35.0-80.0)	P=0.012*
Distant metastasis						
• Absent	27	100	45(17-85)		40(15-80)	
Recurrence						
• Negative	16	59.3	45.0(17.0-85.0)	Z=1.3	42.5(15.0-80.0)	Z=1.12
• Positive	11	40.7	30.0(17.0-80.0)	P=0.17	40.0(15.0-70.0)	P=0.26
TNM grading:						
• TNM1&2	11	40.759.2	20.0(17.0-45.0)	Z=3.3	24.0(15.0-80.0)	Z=2.14
• TNM3&4	16		52.5(20.0-85.0)	P=0.001**	45.0(20.0-80.0)	P=0.03*

KW: Kruskal Wallis test ***P: Probability***

**** Statistically significant if P<0.05*** *****High statistically significant if P<0.01***

^{AB} ***Similar letters denote a significant difference between groups***

cutoff value of 45%, the fascin < 45% (n=12) and fascin ≥ 45 % (n=15) groups, and compared their clinicopathological features. No significant correlation was found between a median fascin cutoff value and age, sex, site, and recurrence. There was a significant association between the median cutoff value of fascin and well differentiated tumor (P=0.04), T3&T4 tumor sizes (P= 0.01), LN infiltration (P=0.05), and TNM3 &TNM4 staging (P=0.02).

Table 3 shows associations between the clinicopathological features of oral squamous cell carcinoma and median cutoff value of WT1. We divided the patients into two groups using a WT1 cutoff value of 40%, the WT1 < 40 % (n=11) and WT1 ≥ 40 % (n=16) groups, and compared their clinicopathological features. There was only a significant association between the median cutoff value of WT1 and moderately differentiated tumor (P=0.02), and LN infiltration (P=0.01).

TABLE (2) Associations between the clinicopathological features of oral aquamous cell carcinoma and fascin (median cutoff value):

	Fascin		Test of significance
	< Median N=12 (%)	≥Median N=15(%)	
Age			
• <60	6(50.0)	10(66.7)	χ^2=0.76
• ≥60	6(50.0)	5(33.3)	P=0.38
Sex			
• Male	7(58.3)	13(86.7)	χ^2=2.78
• Female	5(41.7)	2(13.3)	P=0.09
Site			
• Buccal	4(33.3)	2(13.3)	FET P=0.35
• Gingival	0(0.0)	3(20.0)	FET P=0.23
• Lip	1(8.3)	2(13.3)	FET P=1.0
• Palate	2(16.7)	0(0.0)	FET P=0.19
• Retro	0(0.0)	1(6.7)	FET P=1.0
• Tongue	5(41.7)	7(46.7)	χ^2=0.07 P=0.7
Grade			
• Poor	1(8.3)	6(40.0)	χ^2=3.48 P=0.06
• Well	7(58.3)	3(20.0)	χ^2=4.2 P=0.04*
• Moderate	4(33.3)	6(40.0)	χ^2= 0.13 P=0.7
Tumour size			
• T1&T2	9(75.0)	4(26.7)	χ^2=6.24
• T3&T4	3(25.0)	11(73.3)	P=0.01*
Lymph node involved			
• N0	10(83.3)	7(46.7)	χ^2=3.8
• N1	2(16.7)	8(53.3)	P=0.05*
Recurrence			
• Negative	5(41.7)	11(68.8)	χ^2=2.77
• Positive	7(58.3)	4(26.7)	P=0.09
TNM			
• TNM1&TNM2	8(66.6)	3(20.0)	FET
• TNM3 &TNM4	4(33.3)	12(80.0)	P=0.02*

The median was considered as an arbitrary cutoff point (median value =45)

χ2:Chi-Square test FET: Fischer exact test P:probability* Statistically significant if p≤0.05

TABLE (3) Associations between the clinicopathological features of oral aquamous cell carcinoma and WT1 (median cutoff value).

	WT1		Test of significance
	< Median N=11 (%)	≥Median N=16(%)	
Age			
• <60	5(45.5)	11(68.8)	χ^2=1.46
• ≥60	6(54.5)	5(31.2)	P=0.23
Sex			
• Male	7(63.6)	13(81.2)	FET
• Female	4(36.4)	3(18.8)	P=0.39
Site			
• Buccal	3(27.3)	3(18.8)	FET P=0.66
• Gingival	1(9.1)	2(12.5)	FET P=1.0
• Lip	1(9.1)	2(12.5)	FET P=1.0
• Palate	1(9.1)	1(6.2)	FET P=1.0
• Retro	0(0.0)	1(6.2)	FET P=1.0
• Tongue	5(45.5)	7(43.8)	χ^2= 0.38 P=0.53
Grade			
• Poor	1(9.1)	6(37.5)	χ^2=2.7 P=0.09
• Well	7(63.6)	3(18.8)	χ^2= 5.1 P=0.02*
• Moderate	3(27.3)	7(43.8)	χ^2=0.75 P=0.38
Tumor size			
• T1&T2	7(63.6)	6(37.5)	χ^2=1.78
• T3&T4	4(36.4)	10(62.5)	P=0.18
Lymph node involved			
• N0	10(90.9)	7(43.8)	χ^2=6.2
• N1	1(9.1)	9(56.2)	P=0.01*
Recurrence			
• Negative	6(54.5)	10(62.5)	χ^2=0.17
• Positive	5(45.5)	6(37.5)	P=0.68
TNM			
• TNM1 &TNM2	7(63.6)	4(25.0)	FET
• TNM3 &TNM4	4(36.4)	12(75.0)	P=0.06

The median was considered as an arbitrary cutoff point (median value =40)

χ^2:Chi-Square test FET: Fischer exact test P: probability

**** Statistically significant if p≤0.05***

ROC curve for WT1 and fascin were conducted for detection of lymph node infiltration. Fascin showed excellent AUC (AUC=0.865), with sensitivity of 70.0 % and specificity of 94.1% at cutoff value of 52.5%. WT1 also showed excellent AUC (AUC=0.791), with sensitivity of 70.0% and specificity 70.6% at a cutoff value of 42.2 (figure 1).

ROC curve for WT1 and fascin were conducted for detection of recurrence. Fascin showed fair AUC (AUC=0.66), with sensitivity of 63.6% and specificity of 50.0% at a cutoff value of 45 %. WT1 also showed fair AUC (AUC=0.63), with sensitivity of 63.6% and specificity 68.8 % at a cutoff value of 40% (figure 2).

A log rank test was carried out to determine if

there were differences in the 2 year survival rate of patients with fascin (figure 3) and WT1 (figure 4) expression below and above the median value. The 2-year survival rate of patients with fascin of ≥ 45% and WT1 ≥ 40% were nonsignificantly higher than that of patients with fascin of < 45% and WT1 <40% (P=0.09, P=0.55).

Furthermore, we assessed which factors of age, gender, site, tumor size, histological type, fascin distribution, and WT1 distribution influenced lymph node infiltration. In univariate analysis of lymph node metastasis, fascin, and WT1 were found to be significant risk factors of LN infiltration (p=0.005, 0.02). Multivariate analysis with variables whose p-value were less than 0.15 in univariate analysis demonstrated that any of them can be considered as multivariant risk factors of lymph node metastasis (Table 4). In univariate analysis of risk factors of tumor recurrence, there was no significant risk factor of tumor recurrence (Table 5).

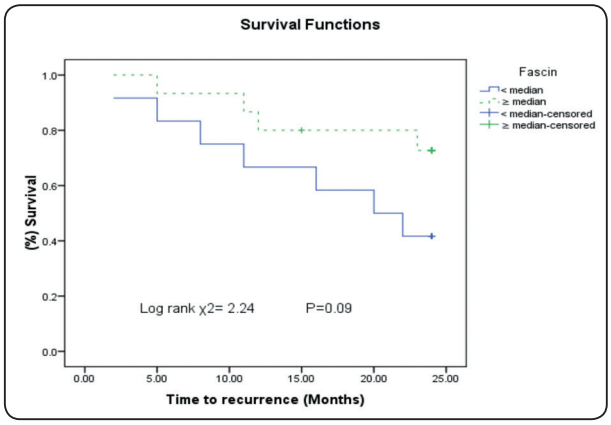

Fig. (3) Kaplan Meier curve for 2 survival analysis of patients with oral squamous cell carcinoma according to Fascin

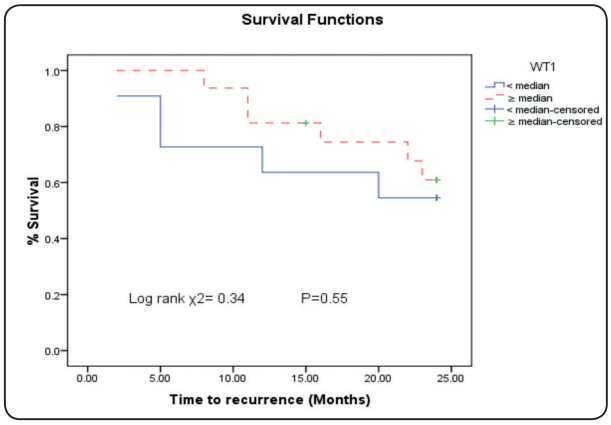

Fig. (4) Kaplan Meier curve for 2 year survival analysis of patients with oral squamous cell carcinoma according to WT1

TABLE (4) Results of univariant analysis and multivariant analyses of lymph node infiltration using binary logistic regression analyses:

Predictors of node infiltration	Univariate analysis		Multivariate analysis	
	P	Odds ratio (95% CI)	P	Odds ratio (95% CI)
Fascin • < median • ≥ median	0.005**	1.09(1.03-1.16)	0.77	0.99(0.92-1.07)
WT 1 • < median • ≥ median	0.02*	1.05(1.01-1.1)	0.74	1.01(0.95-1.07)
Age • < 60 • ≥ 60 (r)	0.38	2.07 (0.39-10.85) 1		
Sex • Male (r) • Female	0.71	1 1.39 (0.24-8.07)		

TABLE (5) Results of univariant analysis of recurrence using binary logistic regression analyses

Predictors of recurrence status	Univariate analysis	
	P	Odds ratio (95% CI)
Fascin <median ≥median	0.45	0.98 (0.92-1.04)
WT 1 <median ≥median	0.87	0.99 (0.95-1.05)
Age <60 ≥60 (r)	0.68	0.64 (0.08-5.42) 1
Sex • Male (r) • Female	0.99	Undefined
T –stage • T1& T2(r) • T3& T4	0.27	1 0.26(0.02-2.85)
Node infiltration • Negative(r) • Positive	0.56	1 1.88(0.22-15.93)

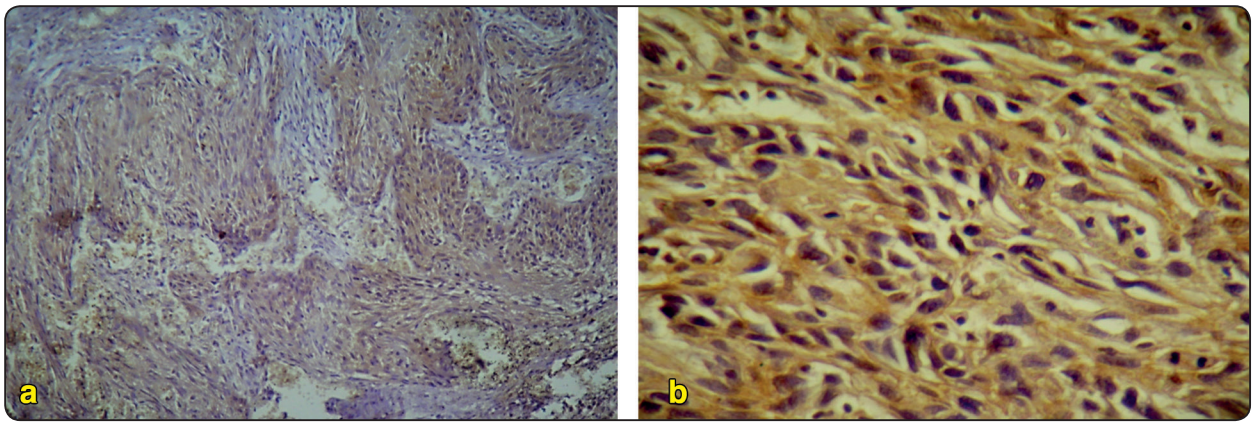

Fig. (5) Photomicrograph shows a) moderate cytoplasmic immuno-reaction with fascin in moderately differentiated OSCC (DAP& X-200), b) intense cytoplasmic immunoreaction with fascin in poorly differentiated OSCC (DAP&X-400).

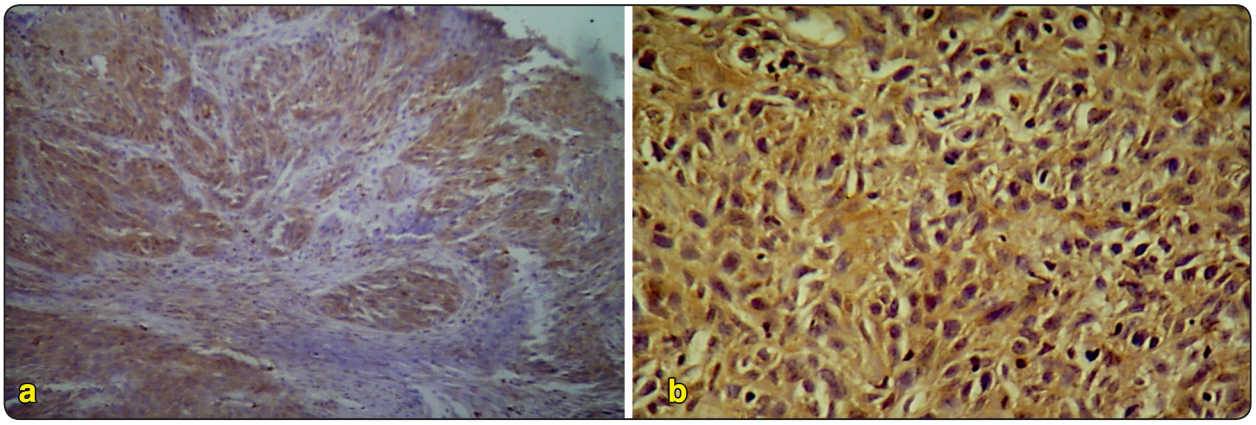

Fig. (6) Photomicrograph shows a) moderate cytoplasmic immunoreaction of WT1 in moderately differentiated OSCC (DAP-X-200), b) intense cytoplasmic immunoreaction with WT1 in poorly differentiated OSCC reveals (DAP&X-400).

DISCUSSION

In this study, fascin was overexpressed in OSCC cells compared with those in non-neoplastic epithelium. This result was coincidental with previous reports that detect increased fascin expression in human carcinomas [15, 25-26]. The increased fascin expression in cancer cells can be explained by A wnt signalling pathway of fascin activity through inactivation of the APC gene or stabilization of b-catenin mutations [27]. Another possible pathway, involving fascin up-regulation in breast cancer cell lines, is dependent on amplification or overexpression of c-erbB-2/HER-2 [28].

As fascin is highly overexpressed in several tumor types, it is also possible that it is secreted by the tumor microenvironment and facilitate tumor development and progression. Transforming Growth Factor ß (TGF-ß) is secreted by the tumor microenvironment and increases fascin expression by phosphorylation of the Smad3 linker region [29]. Prostaglandins are transient bioactive lipids that are also often misregulated in cancer and can influence the adhesive, migratory and invasive potential of cancer cells [30, 31]. Prostaglandins have the potential to regulate the translocation of fascin in and out of the nucleus [32], which might represent another important mechanism to control fascin dependent behavior in tumors.

The distribution of fascin expression was correlated significantly with tumor's histological grades. The fascin distribution was significantly higher in poorly differentiated OSCC than well differentiated and moderately differentiated OSCC. There was a significant association between the median cutoff value of fascin and well differentiated tumor. This result was similar to Alan study, who detects higher fascin expression in higher stages of SCC, however, they don't detect fascin in any of the well differentiated OSCC [16].

Furthermore, this study showed that the median fascin cutoff value was correlated significantly with the most important clinicopathological factors, including the size of the tumor, lymph node metastasis, and clinical TNM stage. Fascin cutoff value was found to be an univariant risk factor for LN metastasis but not for recurrence. The 2-year recurrence free survival rate of patients with a fascin of ≥ 45 % was higher than that of patients with a fascin of < 45 %, however, it is not significant. This result was coincidental with other studies that detected positive correlation between fascin and tumor size [15], tumor stage, N metastasis [15, 16,25], increased recurrence and decrease patients' survival [16,25, 33]

In mesenchymal and also epithelial cells, expression of fascin induces protrusions and increases motility [34, 35]. Previous in vitro studies demonstrated

that elevated levels of fascin increased the speed of cell migration in urothelial carcinoma [36]. Fascin has been shown to promote invasiveness of the colon, breast, esophageal carcinoma derived cells [13, 37, 38] and OSCC [16].

Furthermore, disruption of endogenous fascin expression in nasopharyngeal carcinoma cells suppressed its invasiveness, decreased cell filopodia and lamellipodia, thus, indicating the relevance of fascin to cancer cell invasiveness [33]. Chen et al. found that fascin activity is blocked by migrastatin analogues, decreasing tumor migration, invasion and metastasis in breast cancer [39].

Moreover, fascin-overexpressed cells also demonstrated an increase in MMP-2 activity [16, 40]. MMP-2 and MMP-9 are proteolytic enzymes that digest the components of the basement membrane facilitating metastasis of malignant tumors [41]. During cell migration, thrombpspondin-1 induces cross-linking of fascin and F-actin that leads to formation of F actin based cell protrusions. Fibronectin triggers phosphorylation of fascin at S39, and subsequent loss of F-actin bundling facilitate cell adhesion to fibronectin, laminin and other ECM molecules in OSCC cells, increasing the tumorigenicity of cancer cells [42,43]. Moreover, loss of F-actin bundling resulting in a more diffuse cytoplasmic distribution of fascin as observed in our study and Shimamura et al study [26].

The recycling endosomal protein Rab35, a member of the Rab family of GTPases, has been proposed as one potential regulatory protein involved in transporting fascin to the cell periphery in mammalian cells. In fibroblasts, Rab35 is enriched near the plasma membrane and colocalises with fascin in filopodia, microspikes and lamellipodia. Overexpression of dominant-negative Rab35 limits the presence of fascin at the plasma membrane and increases cytoplasmic accumulation. Disruption of this regulatory mechanism occurs in cancer cell, increasing cytoplasmic distribution [44].

As fascin cutoff value was found to be an univariant risk factor for LN metastasis and high fascin expression was seen in 17% cases where lymph node metastasis was not detected (N0). It will be mandatory to follow these cases further to assess whether fascin expression acts as an indicator "submicroscopic" metastasis, and have an effect on patient survival.

In this study, WT1 was found to have a significant increase in OSCC cells more than the control. This result was in consistence with other studies that registered overexpression of the wildtype WT1 gene in both leukemia and solid tumors [45-48], including OSCC. WT1 mRNA was found overexpressed in one of the six OSCC cell lines and the normal mucosal epithelium did not express WT1 [21]. Oji et al. [19] study, the cutoff levels of WT1 expression levels in normal-appearing mucosal tissues of patients were set at mean + 2 SD; however, muscle cell, myoepithelial cells and endothelial lining cells of blood capillaries also showed WT1 protein and mRNA, and endothelial cells of capillaries proliferate greated in tumor than in an intact mucosa. Langman et al. [20] reported that the normal myoepithelium of the salivary gland was negative for WT1 protein, the neoplastic myoepithelium in pleomorphic adenomas of the salivary gland was positive for it.

There was correlation between WT1 and histologic differentiation, significant difference between WT1 of poorly differentiated and well differentiated OSCC (P= 0.003). This result was in consistence with Mikami et.al, WT1 protein was detected on actively proliferating cancer nests and was also expressed in the prickle cell layers of epithelium where cell adhesion was weakened to form tumor nests for invasion [21]. Oji et al. [19] reported that high expression levels of the WT1 gene showed significant correlation with poor histologic tumor differentiation and advanced tumor stage of head and neck SCC. WT1 is expressed in lower differentiated epithelium instead of higher differentiated epithelium during the process

of tumorigenic transformation and neoplastic cells [20]. On the other hand, only one study stated that there was not a significant relation between histopathological grade and WT1 expression [49].

In this study, overexpression of WT1 concentrated mainly in the cytoplasm. This is in agreement with Li et al who found 5 samples of tumor samples in SCC of head and neck showed positive staining in cytoplasm [50]. Nakatsuka et al examined 494 cases of different human neoplasias including tumors of the urinary and gastrointestinal tract, female and male genital organs, lung, skin, breast, brain, soft tissues and bone using an immunohistochemical approach. A majority of the positive cases showed diffuse or granular staining in the cytoplasm. Also the cytoplasmic expression of WT1 in ameloblastoma [51-53].

These results can be explained by Li et al. who found that WT1 and p63 promoted cell proliferation. In addition of expression of 18 genes involved in cell proliferation, cell cycle regulation and DNA replication were significantly altered by downregulation of WT1. Several known WT1 and p63 target genes were affected by WT1 knockdown. Additionally, high WT1 mRNA levels were detected in SCC of head and neck patient samples. WT1 and p63 are involved in cancer cell growth [51].

In this study, WT1 Distribution was significantly higher in OSCC associated with lymph node infiltration than OSCC without LN infiltration. Moreover, OSCC with TNM3&4 showed a significant WT1 distribution than OSCC with TNM1&2. There was a significant association between the median cutoff value of WT1 and moderately differentiated tumor (P=0.02), and LN infiltration (P=0.01). These results in agreement with Li et al. [54] who found the nuclear and cytoplasmic expression of WT1- associated protein in tumor tissues was significantly higher than non-tumor tissues (P<0.001). An univariate analysis revealed that high nuclear expression of WTAP was significantly associated with poor overall survival P<0.001, and Oji et al. [19] reported that WT1 mRNA expression was correlated with the tumor stage of head and neck squamous cell carcinoma. Overexpression of WT1 enhanced the ability of cell proliferation and invasion. High levels of WT1 expression were associated with lymph node and omentum metastasis of ovarian cancer patients. WT1 was highly expressed in aggressive carcinomas and carcinosarcoma. These results were obtained using a real-time quantitative PCR (qPCR) method to examine the precise quantification of WT1 expression levels in clinical samples. WT1 is highly expressed in patients with higher-stage cancers, lymph node and omentum metastasis, and ascites production [55] . This can be explained as WT1 has been found to be a potent transcriptional regulator of genes important for cellular growth and metabolism, including extracellular matrix components, growth factors, and other transcription factors [56]. WT1 was found to bind to the promoter of a tumor suppressor gene CDC73, negatively regulate its activity, and promote the proliferation of OSCC cells [57]. WT1 could also promote invasion, migration and metastasis [58-60], the association between WT1 expression in breast cancer and poor prognosis is potentially due to cancer-related epithelial-to-mesenchymal transition (EMT) and facilitate angiogenesis [61,62].

No statistically significant differences in WT1 immunohistochemical staining were observed by clinical variables such as age, sex, location, size in agreement with Li et al and Bolonga et al [50,53].

Because WT1 has been identified as a molecular target for cancer immunotherapy, immunohistochemical detection of WT1 in tumor cells has become essential [51]. Two separate phase I clinical studies by Tsuboi et al. [63] and Oka et al. [64] using WT1 peptide-based cancer immunotherapy in patients with cancers of same histologic types as OSCC (i.e., squamous cell carcinoma) and that also express WT1 proteins both demonstrated a decrease in tumor marker expression and were considered to be effective. Recent clinical studies have demonstrated

that patients with hematological malignancies who have minimal residual disease after therapy may be cured by WT1 peptide vaccination [65]. In a phase II clinical study showing that WT1 vaccination could induce WT1-specific cytotoxic T lymphocytes and cancer regression without damage to normal tissues[64,66]. Thus, these findings suggest WT1 peptide-based immunotherapy to be a new treatment option for OSCC.

LIMITATION OF THE STUDY

Some limitations of this retrospective study are present, such as the proper match of the control group; and the possible confounding factors that may affect the results. Also, the sample size was small.

CONCLUSION

Fascin and WT1 have oncogenic effects. Overexpression of them contributes to a more aggressive clinical course. Understanding the role of them on OSCC and other tumors will facilitate the development of new treatment strategies.

RECOMMENDATION

Larger multicenter studies are needed to confirm the oncogenic effects of fascin and WT1. Also, subsequent studies of protein expression in cell lines of OSCC by Western blot analysis and vector-based siRNA by semiquantitative reverse transcriptase-polymerase chain reaction are now underway, designed to study the effects of down-regulation of fascin and WT1 for help in cancer treatment.

REFERENCES

1. Parkin DM, Bray F, Ferlay J, and Pisani P. Global cancer statistics, 2002. CA Cancer J Clin 2005, 55:74-108.
2. Rusthoven K, Ballonoff A, Raben D, and Chen C. Poor prognosis in patients with stage I and II oral tongue squamous cell carcinoma. Cancer 2008;112:345-351.
3. Mucke T, Wagenpfeil S, Kesting MR, Holzle F, and Wolff KD. Recurrence interval affects survival after local relapse of oral cancer. Oral Oncol 2009; 45:687-691.
4. Liotta LA, and Kohn EC. The microenvironment of the tumor-host interface. Nature 2001;411: 375–379.
5. Matsudaira P. Actin crosslinking proteins at the leading edge. Semin Cell Biol 1994; 5: 165–174.
6. Kureishy N, Sapountzi V, Prag S, Anilkumar N, and Adams JC. Fascins, and their roles in cell structure and function. Bioessays 2002; 24:350-361.
7. Cohan CS, Welnhofer EA, Zhao L, Matsumura F, and Yamashiro S. Role of the actin bundling protein fascin in growth cone morphogenesis: localization in filopodia and lamellipodia. Cell Motil Cytoskeleton 2001;48:109-120.
8. Goncharuk VN, Ross JS, and Carlson JA. Actin binding protein fascin expression in skin neoplasia. J Cutan Pathol 2002;29:430–438.
9. Machesky LM, and Li A. Fascin: Invasive filopodia promoting metastasis. Commun Integr Biol 2010; 3:263-270.
10. Alam H, Kundu ST, Dalal SN, and Vaidya MM. Loss of keratins 8 and 18 leads to alterations in alpha6 beta4-integrin-mediated signalling and decreased neoplastic progression in an oral-tumour-derived cell line. J Cell Sci 2011; 124:2096-2106.
11. Pelosi G, Pasini F, Fraggetta F, Pastorino U, Iannucci A, Maisonneuve P, Arrigoni G, De Manzoni G, Bresaola E, and Viale G. Independent value of fascin immunoreactivity for predicting lymph node metastases in typical and atypical pulmonary carcinoids. Lung Cancer 2003, 42:203-213.
12. Hashimoto Y, Skacel M, Lavery IC, Mukherjee AL, Casey G, and Adams JC. Prognostic significance of fascin expression in advanced colorectal cancer: an immunohistochemical study of colorectal adenomas and adenocarcinomas. BMC Cancer 2006, 6:241.
13. Zigeuner R, Droschl N, Tauber V, Rehak P, and Langner C. Biologic significance of fascin expression in clear cell renal cell carcinoma: systematic analysis of primary and metastatic tumor tissues using a tissue microarray technique. Urology 2006; 68:518-522.
14. Chen SF, Yang SF, Li JW, Nieh PC, Lin SY,Fu E, Bai CY, Jin JS, Lin CY, and Nieh S. Expression of fascine in oral and oropharyngeal squamous cell carcinomas has prognostic significance - a tissue microarray study of 129 cases. Histopathol 2007; 51:173-183.
15. Alam H, Bhate AV, Gangadaran P, Sawant SS, Salot S, Sehgal L, Dange PP, Chaukar DA, D'cruz AK, Kannanl S, Gude R, Kane S, Dalal SN, and Vaidya MM. Fascin overexpression promotes neoplastic progression in oral squamous cell carcinoma. BMC Cancer 2012, 12:32.

16. Morrison AA, Viney RL, and Ladomery MR. The posttranscriptional roles of WT1, a multifunctional zinc finger protein. Biochim Biophy Acta 2008; 1785:55-62.

17. Shandilya J, Toska E, Richard DJ, Medler KF, and Roberts SG. WT1 interacts with MAD2 and regulates mitotic checkpoint function. Nat Commun 2014; 5:4903.

18. Chau YY, and Hastie ND. The role of Wt1 in regulating mesenchyme in cancer, development, and tissue homeostasis. Trends Genet 2012; 28:515-524.

19. Oji Y, Inohara H, Nakazawa M, Nakano Y, Akahani S, Shin-ichi Nakatsuka, Koga S, Ikeba A, Abeno S, Honjo Y,Yamamoto Y, Iwai S, Yoshida K, Oka Y,Ogawa H, Yoshida J, Aozasa K, Kubo T, and Sugiyama H. Overexpression of the Wilms' tumor gene WT1 in head and neck squamous cell carcinoma. Cancer Sci 2003; 94: 523–529.

20. Langman G, Andrews CL, and Weissferdt A. WT1 expression in salivary gland pleomorphic adenomas: a reliable marker of the neoplastic myoepithelium. Mod Pathol 2011; 24: 168–174.

21. Mikami T, Hada T, Chosa N, Ishisaki A, Mizuki H, and Takeda Y. Expression of Wilms' tumor 1 (WT1) in oral squamous cell carcinoma J Oral Pathol Med 2013; 42: 133–139.

22. Sobin L, Gospodarowicz M and Wittekind C (eds.). TNM Classification of Malignant Tumors. Seventh edition. Hoboken, NJ: John Wiley & Sons, Inc., 2009.

23. Schuon R, Brieger J, Heinrich UR, Roth Y, Szyfter W, and Mann WJ. Immunohistochemical analysis of growth mechanisms in juvenile nasopharyngeal angiofibroma. Eur Arch Otorhinolaryngol 2007; 264: 389-394.

24. Bittinger F, Brochhausen C, Köhler H, Lehr HA, Otto M, Skarke C, Walgenbach S, and Kirkpatrick CJ. Differential expression of cell adhesion molecules in inflamed appendix: correlation with clinical stage. J Pathol. 1998;186:422–428.

25. Lee TK, Poon RTP, Man K, Guan XY, Ma S, Liu XB, Myers JN, and Yuen APW. Fascin over-expression is associated with aggressiveness of oral squamous cell carcinoma. Cancer Lett. 2007;254:308–315.

26. Shimamura Y, Abe T, Nakahira M, Yoda T, Murata S, and Sugasawa M. Immunohistochemical Analysis of Oral Dysplasia: Diagnostic Assessment by Fascin and Podoplanin Expression Acta Histochem. Cytochem. 2011; 44 (6): 239–245.

27. Tao YS, Edwards RA, Tubb B, Wang S, Bryan J, and McCrea PD. b-Catenin associates with the actin-bundling protein fascin in a non-cadherin complex. J Cell Biol 1996; 134: 1271–1281.

28. Grothey A, Hashizume R, Ji H, Tubb BE, Patrick CW, Yu D, Mooney E, and McCrea PD. C-erbB-2 / HER-2 upregulates fascin, an actin-bundling protein associated with cell motility, in human breast cancer cell lines. Oncogene 2000; 19: 4864–4875.

29. Li L, Cao F, Liu B, Luo X, Ma X, and Hu Z. TGF-b induces fascin expression in gastric cancer via phosphorylation of smad3 linker area.AmJ Cancer Res 2015; 5:1890-1896.

30. Karnezis T, Shayan R, Fox S, Achen MG, and Stacker SA. The connection between lymphangiogenic signalling and prostaglandin biology: A missing link in the metastatic pathway. Oncotarget 2012; 3:890-903.

31. Menter DG, and DuBois RN. Prostaglandins in cancer cell adhesion, migration, and invasion. Int J Cell Biol 2012; 2012:723419.

32. Groen CM, Jayo A, Parsons M, and Tootle TL. Prostaglandins regulate nuclear localization of Fascin and its function in nucleolar architecture. Mol Biol Cell 2015; 26:1901-1917.

33. Wu D, Chen L, Liao W, Ding Y, Zhang Q, Li Z, and Liu L. Fascin1 expression predicts poor prognosis in patients with nasopharyngeal carcinoma and correlates with tumor invasion. Ann Oncol 2010;21: 589596.

34. Adams JC. Fascin protrusions in cell interactions. Trends Cardiovasc Med 2004;14: 221226.

35. Yamashiro S, Yamakita Y, Ono S, and Matsumura F. Fascin, an actinbundling protein, induces membrane protrusions and increases cell motility of epithelial cells. Mol Biol Cell 1998;9: 9931006.

36. Karasavvidou F, Barbanis S, Pappa D, Moutzouris G, Tzortzis V, Melekos MD, and Koukoulis G. Fascin determination in urothelial carcinomas of the urinary bladder: a marker of invasiveness. Arch Pathol Lab Med 2008;132: 19121915.

37. Jawhari AU, Buda A, Jenkins M, Shehzad K, Sarraf C, Noda M, Farthing MJ, Pignatelli M, and Adams JC. Fascin, an actin-bundling protein, modulates colonic epithelial cell invasiveness and differentiation in vitro. Am J Pathol 2003, 162:69-80.

38. Hwang JH, Smith CA, Salhia B, and Rutka JT. The role of fascin in the migration and invasiveness of malignant glioma cells. Neoplasia 2008, 10:149-159.

39. Chen L, Yang S, Jakoncic J, Zhang JJ, and Huang XY. Migrastatin analogues target fascin to block tumour metastasis. Nature 464:1062-1066.

40. Xie JJ, Xu LY, Zhang HH, Cai WJ, Mai RQ, Xie YM, Yang ZM, Niu YD, Shen ZY, and Li EM. Role of fascin in the proliferation and invasiveness of esophageal carcinoma cells. Biochem Biophys Res Commun 2005, 337:355-362.

41. Liotta LA, Tryggvason K, Garbisa S, Hart I, Foltz CM, and Shafie S. Metastatic potential correlates with enzymatic degradation of basement membrane collagen. Nature 1980;284:67-68.

42. Adams JC. Formation of stable microspikes containing actin and the 55 kDa actin bundling protein, fascin, is a consequence of cell adhesion to thrombospondin-1: implications for the anti-adhesive activities of thrombospondin-1. J Cell Sci 1995, 108(Pt 5):1977-1990.

43. Adams JC, Clelland JD, Collett GD, Matsumura F, Yamashiro S, and Zhang L. Cell-matrix adhesions differentially regulate fascin phosphorylation. Mol Biol Cell 1999; 10:4177-4190.

44. Zhang J, Fonovic M, Suyama K, Bogyo M, and Scott MP. Rab35 controls actin bundling by recruiting fascin as an effector protein. Science (New York, NY) 2009; 325: 1250-1254.

45. Oji Y, Ogawa H, Tamaki H, Oka Y, Tsuboi A, Kim EH, Soma T, Tatekawa T, Kawakami M, Asada M, Kishimoto T, and Sugiyama H. Expression of the Wilms' tumor gene WT1 in solid tumors and its involvement in tumor cell growth. Jpn J Cancer Res 1999; 90, 194–204.

46. Osaka M, Koami K, and Sugiyama T. WT1 contributes to leukemogenesis: expression patterns in 7,12-dimethylbenz[a]anthracene (DMBA)-induced leukemia. Int J Cancer 1997; 72, 696–699.

47. Inoue K, Tamaki H, Ogawa H, Oka Y, Soma T, Tatekawa T, Oji Y, Tsuboi A, Kim EH, Kawakami M, Akiyama T, Kishimoto T, and Sugiyama H. Wilms' tumor gene (WT1) competes with differentiation gene (WT1) is expressed in primary breast tumors despite tumor specific inducing signal in hematopoietic progenitor cells. Blood 1998; 91, 2969–2976.

48. Loeb DM, Evron E, Patel CB, Sharma PM, Niranjan B, Buluwela L, Weitzman SA, Korz D, and Sukumar S. Wilms' tumor suppressor promoter methylation. Cancer Res. 2001;61, 921–925.

49. Fattahi S, Rahmani SZ, Vosoughhosseini S, and Rahmani SP. Configuring the Expression of Wilms Tumor 1 in Oral Squamous Cell Carcinoma and Its Relationship with Clinicopathologic Features. J Dent App 2016; 3(4):349-352.

50. Li X, Ottosson S, Wang S, Jernberg E, Boldrup L, Gu X, Nylander K, and Li A. Wilms' tumor gene 1 regulates p63 and promotes cell proliferation in squamous cell carcinoma of the head and neck. BMC Cancer 2015 May 1;15:342.

51. Nakatsuka S, Oji Y, Horiuchi T, Kanda T, Kitagawa M, Takeuchi T, Kawano K, Kuwae Y, Yamauchi A, Okumura M, Kitamura Y, Oka Y, Kawase I, Sugiyama H, and Aozasa K. Immunohistochemical detection of WT1 protein in a variety of cancer cells. Mod Pathol 2006;19,804-814.

52. Benavides PZ, Rodríguez MA, Goytia JJ, Molina MA, Ochoa GA, Díaz EA, Esparza RH, and Padilla CR. Cytoplasmic Localization of WT1 and Decrease of miRNA-16-1 in Nephrotic Syndrome. Biomed Res Int 2017;2017:9531074.

53. Bologna RM, Takeda Y, Kuga T, Chosa N, Kitagawa M, Takata T, Ishisaki A, and Mikami T. Expression of Wilms' tumor 1 (WT1) in ameloblastomas. J Oral Sci. 2016;58(3):407-413.

54. Li B, Huang SH, Shao Q, Sun J, Zhou L You, Zhang L, Liao Q, Guo J, and Zhao Y. WT1-associated protein is a novel prognostic factor in pancreatic ductal adenocarcinoma Oncology Letters 2017;13: 2531-2538.

55. Liu Z , Yamanouchi K, Ohtao T, Matsumura S, Seino M, Shridhar V, Takahashi T, Takahashi K, and Kurachi H. High levels of Wilms' tumor 1 (WT1) expression were associated with aggressive clinical features in ovarian cancer. Anticancer Res 2014;34: 2331–2340.

56. Høgdall EV Christensen L, Kjaer SK, Blaakaer J, Christensen IJ, Gayther S, Jacobs IJ, and Høgdall CK. Expression level of Wilms tumor 1 (WT1) protein has limited prognostic value in epithelial ovarian cancer: from the Danish «MALOVA» ovarian cancer study. Gynecol Oncol 2007;106: 318–324.

57. Rather MI, Swamy S, Gopinath KS, and Kumar A. Transcriptional Repression of Tumor Suppressor CDC73, Encoding an RNA Polymerase II Interactor, by Wilms Tumor 1 Protein (WT1) Promotes Cell Proliferation. J Biol Chem 2014 Jan 10;289(2):968-976.

58. Wu C, Zhu W, Qian J, He S, Wu C, Chen Y, and Shu Y. WT1 Promotes Invasion of NSCLC via Suppression of CDH1. J Thorac Oncol 2013; 8: 1163–1169.

59. Barbolina MV, Adley BP, Shea LD, and Stack MS. Wilms tumor gene protein1 is associated with ovarian cancer metastasis andmodulates cell invasion. Cancer 2008;112: 1632–1641.

60. Brett A, Pandey S, and Fraizer G. The Wilms' tumor gene (WT1) regulates Ecadherin expression and migration of prostate cancer cells. Mol Cancer 2013;12: 3.

61. Artibani M, Sims A H., Slight J, Aitken S, Thornburn A, Muir M, Brunton VG, Del-Pozo J, Morrison LR, Katz E, Hastie ND, Hohenstein P. WT1expression in breast cancer disrupts the epithelial/ mesenchymal balance of tumor cells and correlates with the metabolic response to docetaxel. Scientific RepoRts 2017; 7:45255.

62. Katuri V, Gerber S, Qiu X, McCarty G, Goldstein SD, Hammers H, Montgomery E, Chen AR, and Loeb DM. WT1 regulates angiogenesis in Ewing Sarcoma. Oncotarget 2014;5: 2436–2449.

63. Tsuboi A, Oka Y, Osaki T, Kumagai T, Tachibana I, Hayashi S, Murakami M, Nakajima H, Elisseeva OA, Fei W, Masuda T, Yasukawa M, Oji Y, Kawakami M, Hosen N, Ikegame K, Yoshihara S, Udaka K, Nakatsuka S, Aozasa K, Kawase I, and Sugiyama H. WT1 peptide-based immunotherapy for patients with lung cancer: report of two cases. Microbiol Immunol 2004; 48(3): 175–184.

64. Oka Y, Tsuboi A, Taguchi T, Osaki T, Kyo T, Nakajima H, Elisseeva OA, Oji Y, Kawakami M, Ikegame K, Hosen N, Yoshihara S, Wu F, Fujiki F, Murakami M, Masuda T, Nishida S, Shirakata T, Nakatsuka S, Sasaki A, Udaka K, Dohy H, Aozasa K, Noguchi S, Kawase I, and Sugiyama H. Induction of WT1 (Wilms' tumor gene)-specific cytotoxic T lymphocytes by WT1 peptide vaccine and the resultant cancer regression. Proc Natl Acad Sci U S A. 2004 Sep 21;101(38):13885-13890.

65. Oka Y, Tsuboi A, Nakata J, Nishida S, Hosen N, Kumanogoh A, Oji Y, Sugiyama H. Wilms' Tumor Gene 1 (WT1) Peptide Vaccine Therapy for Hematological Malignancies: From CTL Epitope Identification to Recent Progress in Clinical Studies Including a Cure-Oriented Strategy. Review Article Oncol Res Treat 2017;40:682–690.

66. Oka Y, Elisseeva OA, Tsuboi A, Ogawa H, Tamaki H, Li H, Oji Y, Kim EH, Soma T, Asada M, Ueda K, Maruya E, Saji H, Kishimoto T, Udaka K, and Sugiyama H. Human cytotoxic T-lymphocyte responses specific for peptides of the wild-type Wilms' tumor gene (WT1) product. Immunogenetics 2000; 51: 99–107.