Variation in Immunohistochemical Expression of Neuropilin1 among Oral, Laryngeal and Skin SCC

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ABSTRACT

Background: Neuropilin 1(NRP1) is considered a novel non - tyrosine kinase co- receptor for the vascular endothelial growth factors (VEGF). First discovered on migrating neurons. NRP1is suggested to be up-regulated in cells of different types of cancer and implicated with advanced disease. The aim of this study was to investigate the variation in expression of NRP1 in oral, laryngeal and skin squamous cell carcinoma.

Materials and methods: Tissue sections from 120 formalin fixed- paraffin embedded blocks histopathologically diagnosed as oral, laryngeal and skin SCC (40 blocks for each),immunohistohemically stained in immunoperoxidase method with monoclonal antibodies to NRP1, the localization of expression was examined and the resulting scores were analyzed according to age, sex, and histopathological grades.

Results: The immunohistochemical analysis revealed that the NRP1 expression in oral, laryngeal and skin squamous cell carcinoma was (87.5%), (92.2%) and (82.5%) respectively, with no significant variation in expression among them(P=0.44), but, NRP1 up-regulation in all the three types correlated positively with degree of differentiation (P=0.009), (P=0.002) and (P=0.007) respectively.

Conclusion: Angiogenesis play an important and similar role in carcinogenesis of oral, laryngeal and skin squamous cell carcinoma, and NRP1 is significantly associated with degree of differentiation in the three types of carcinomaso it can be act as a prognostic marker.

Keywords: Neuropilin-1**,** VEGF, Squamous cell carcinoma, Immunohistochemistry, Expression. **(J Bagh Coll Dentistry 2017; 29(1)):63-69).**

INTRODUCTION

Cancer angiogenesis is a crucial process in growth of tumor as it ensures nutrient and oxygen to the proliferating malignant cells by development of new blood vessels, leading to progression and metastasis of cancer [1]. The progression of the tumor from a non-angiogenic to an angiogenic phenotype is known as the angiogenic switch. This angiogenic switch is triggered by signals like metabolic stress (low pH, low oxygen pressure), mechanical stress, inflammatory response, and genetic mutations [2] .

The vascular endothelial growth factors family involved in process of angiogenesis [3]. VEGFs play an important role in angiogenesis of cancer by stimulating the growth of new blood vessels within the tumor [4]. VEGFs initiate their biological effect by binding to specific tyrosine kinase receptors (VEGFR1, VEGFR2 and VEGFR3) in addition to non- tyrosine kinase co-receptors like neuropilins 1 and 2 [5] .

Neuropilin 1 is a protein which is encoded by NRP1 gene in humans [6]. NRP1 has been implicated in extensive range of functions that range from immunological responses to cell adhesion via interaction with integrins [7]. NRP-1 expression increase stumorigenisity by promoting VEGFs mediated angiogenesis [8]. It is expressed on numerous types of cancerous cells. In several cancers the expression is associated with progression of tumor and/or bad prognosis [9]. High levels of NRP1 is associated with cancer aggressiveness, advanced stage and unfavorable prognosis [10]. Up-regulation of NRP-1 is correlated with invasive behavior and metastatic potential of tumors [11].

MATERIALS AND METHODS

A total of one-hundred and twenty cases of Formalin-fixed, paraffin embedded tissue blocksthat histo-pathologically diagnosed as oral, laryngeal and cutaneous squamous cell carcinoma (fourty blocks for each type) were included in the study. Oral squamous cell carcinoma blocks were collected from the archives of Oral Pathology Department, College of Dentistry, University of Baghdad, while the laryngeal and cutaneous squamous cell carcinoma cases were obtained from histopathology laboratory of Ghazi al Hariry Hospital ofSpecialized Surgeries for the period from October 2014 till June 2015.Immunohistochemical analysis was performed on the samples to evaluate the expression of NRP1. Five Micron thick tissue sections of the blocks were mounted on positively charged slides, dewaxed and rehydrated in xylene

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and serial dilutions of ethanol. Endogenous peroxidase activity and non-specific antibody binding were blocked with H2O2 and protein block respectively. After blocking , the antigens were retrieved in a hot solution (100X Citrate Buffer pH 6.0) for 10 minutes The sections were incubated with a rabbit polyclonal anti-NRP1 antibody [EPR3113] diluted into (1:1000) for 6 hours. Subsequently, biotin free-HRP linked secondary antibodies were applied. Followed by application of diluted DAB (chromogenic solution) onto sections and counterstained with hematoxylin. Immunoreactivity was semi-quantitatively evaluated for positivelystained cells showing immunoreactivity on the cell membrane and/or cytoplasm in five representative microscopic fields. Then calculating the percentage of positive considered cells. The expression of NRP-1 in tissue sections was evaluated 0 when no positive stained cells observed, score 1 (weak) in case of < 30% of tumor cells were positive, score 2(moderate) when 30- 60% of positive cells identified and score 3(strong) when< 60% of tumor cells counted [12]. Statistical analysis was performed using the SPSS version 21 computer software in association with Microsoft Excel. The statistical significance of variations in median was tested via Kruskal Wallis test, and correlations were assessed by Spearman Rank linear correlation coefficient.

RESULTS

Table (1)shows that, most of the cases of oral, laryngeal and skin squamous cell carcinoma the age ranged from 50 to 69 years with (50%) for OSCC, (80%) for LSCC and (47%) for skin SCC. Also, this table showed that most of patients were males in oral, laryngeal and skin SCC, (52.5%), (72.5%) and (67.5%) respectively.

According to table (2),well differentiated grade was the most frequentin OSCC 18 cases (45.0%), followed by moderately differentiated 15 cases (37.5%) and poorly differentiated 7 cases (17.5%). Whereas in LSCC the predominant grade was moderately differentiated 17 cases (42.5%), followed by well differentiated 12 (30.0%)and poorly differentiated 11 cases (27.5%). . In skin the well differentiated degree was so high 24 cases (60.0%) compared to moderately differentiated 11 (27.5%) and Poorly differentiated 5 cases (12.5%).

The pattern of expression of NRP 1 in the present study, was cytoplasmic and/or membranous as shown in figures (1),(2) and (3).

As shown in table(3),105 cases were positively stained with NRP1 Ab in the three types of cancers (87.5%) while 15 cases were negative (12.5%).The immunostaining was distributed equally between score 2 and 3 in oral SCC (14cases) (35.5%) for each , and for LSCC (17 cases) (42.5%).In Skin SCC the positive cases were (33)(82.5%).The predominant score was 3 (16 cases) (40%), followed by score 2 (11cases) (27.5%).The mean rank of median expression of scores for the three types were (57.4%), (65.9%) and (58.2%) respectively with a non-significant difference among them.Tables (4,5 and6) showed that, the median score of NRP1was the lowest among subjects with grade I tumor and increased with increasing tumor grade to reach its highest median score among those with grade III (poorly differentiated) in OSCC, LSCC and Skin SCC with significant correlation (P=0.009),(P=0.002) and (P=0.007) respectively.

DISCUSSION

Statistical analysis of the study results revealed high percentage of NRP1 expression in OSCC, LSCC and Skin SCC (87.5%), (92.5%) and (82.5%) respectively which was consistent with previous evidenced proved results which consider NRP1 being widely up-regulated in neoplastic epithelium compared to normal epithelium or to neoplasms which are not of epithelial origin, like neuroblastomas , glioblatomas and melanomas [13].

Ding et al, (2014) had found no significant correlation of NRP1 expression with both age or gender [14], which is in contrast to study results that showed a significant correlation with OSCC and gender, but no obvious relationship with age.A significant correlation with degree of differentiation was reported in previous researches [14 & 15] and that is similar to the present results (P=0.009) in OSCC, (P=0.002) in LSCC and (P=0.007) in Skin SCC. This positive correlation with histopathological gradeswas proved by one study which stated that angiogenesis in well and moderate differentiated SCC is more than that in non-cancerous epithelium , and in poorly differentiated SCC angiogenesis is much more intense than in well differentiated SCC [16]. The expression of NRP-1 to VEGFR2 increases in association with tumor grade [17],and overexpression of NRP1 is associated with intensive vascularization [18] .

In head and neck SCC, vascular endothelial growth factor A (VEGFA) is the main mediator of angiogenesis [19]. VEGF-A bind to numerous receptors including KDR, FLT1 and NRP1 and induce angiogenesis by activation of kinase cascade which include Ras as well as MAPK [20].

It has been found that in lining epithelium NRP-1 affects TGF-β1 signaling. TGF- β1 is a major

control of epithelial mesenchymal transition (EMT). Epithelial mesenchymal transitionpromoting progression and invasion of malignant cell into surrounding tissue via molecular changes to epithelial cells which promote cell- cell adhesive disfunction[21]. So NRP-1 act as an enhancer of EMT process in HNSCC process [22].

Additionally, NRP1 serves as a regulator of Hedgehog (Hh) signal [23] and target for Shh signaling [24]. So NRP1 is important for mediating VEGF effects on cancer cells [25].

In Skin SCC it has been found that VEGF ligand increases in epidermis with squamous cell carcinomas or when exposed to Ultra violet B (UVB) irradiation. Over-expression of VEGF in low grade SCC rises their growth rate as well invasiveness [15]. Skin cancer cells expressed both endogenous VEGF-A as well as NRP-1 [26],Where NRPs which are co-receptor for VEGF, increasing their activity [27]. Binding of VEGF-A to NRP-1 promoting signaling such as the MAPK pathway and contribute to progression of tumor [28]. VEGF appeared to act as an internal autocrine survival mediator in NRPs positive cancer cells [13].The pattern of expression of NRP 1 in the present study, was cytoplasmic and/or membranous and this in agreement with most previous studies such as Yacoub et al in prostatic cancer [29]; Ding et al in lung cancer [14]. Xu et al, in nasopharnx [30]. This is because NRP1 receptors are mainly found in cytoplasm and membranes of tumor cells [31]. NRP1 have a large extra cellular membrane domain, short transmembrane domain and small cytoplasmic not enzymatic domain [32]. In addition a naturally occurring soluble NRP-1protein (sNRP-1), that containing only part of the extra-cellular domain, generated via alternative splicing of NRP-1 gene [11].

In conclusion, the absence of significance that relating to biological behavior variation among the three types (P=0.44) , despite high expression observed suggesting that angiogenesis plays a crucial and similar role in carcinogenesis in cancer of epithelial in origin, and its positive correlation with degree of differentiation speculating that NRP1 can predict prognosis in OSCC, LSCC and Skin SCC. The prognostic significance of the expression needs to be clarified in further studies.

Table 1: Frequency distribution of the 3 study groups by age and gender.

	Study group					
	Oral SCC		Laryngeal SCC		Skin SCC	
	N	%	N	%	N	%
Age group (years)						
<50	11	27.5	3	7.5	13	32.5
50-69	20	50.0	32	80.0	19	47.5
70+	9	22.5	5	12.5	8	20.0
Total	40	100.0	40	100.0	40	100.0
Gender						
Female	19	47.5	11	27.5	13	32.5
male	21	52.5	29	72.5	27	67.5
Total	40	100.0	40	100.0	40	100.0

Table 2: Frequency distribution of the 3 study groups by tumor grade

	Study group					
	Oral SCC		Laryngeal SCC		Skin SCC	
	N	%	N	%	N	%
Tumor grade						
Well differentiated	18	45.0	12	30.0	24	60.0
Moderately differentiated	15	37.5	17	42.5	11	27.5
Poorly differentiated	7	17.5	11	27.5	5	12.5
Total	40	100.0	40	100.0	40	100.0

Table 3: The difference in median score category of NRP1 among the 3 study groups.

	Study group						
	Oral SCC		Laryngeal SCC		Skin SCC		
	N	%	N	%	N	%	P
NRP1 score							0.44[NS]
Negative (0%)	5	12.5	3	7.5	7	17.5	
Score-1 (1-29%)	7	17.5	3	7.5	6	15.0	
Score-2 (30-60%)	14	35.0	17	42.5	11	27.5	
Score-3 (61%+)	14	35.0	17	42.5	16	40.0	
Total	40	100.0	40	100.0	40	100.0	
Median	Score-2 (30-60%)		Score-2 (30-60%)		Score-2 (30-60%)		
Mean rank	57.4		65.9		58.2		

P (Mann-Whitney) for difference between:
Laryngeal SCC X Oral SCC = 0.23[NS]
Skin SCC X Oral SCC = 0.96[NS]
Skin SCC X Laryngeal SCC = 0.31[NS]

Table 4: The difference in median score category of selected NRP1 marker between the 3 tumor grades among cases with oral SCC.

	Tumor grade						
	Well differentiated		Moderately differentiated		Poorly differentiated		
Oral SCC	**N**	**%**	**N**	**%**	**N**	**%**	**P**
NRP1 score							0.012
Negative (0%)	2	11.1	3	20.0	0	0.0	
Score-1 (1-29%)	4	22.2	3	20.0	0	0.0	
Score-2 (30-60%)	10	55.6	3	20.0	1	14.3	
Score-3 (61%+)	2	11.1	6	40.0	6	85.7	
Total	18	100.0	15	100.0	7	100.0	
Median	Score-2		Score-2		Score-3		
Mean rank	16.9		19.7		31.5		
r=0.41 P=0.009							

Table 5: The difference in median score category of selected NRP1 marker between the 3 tumor grades among cases with Laryngeal SCC.

	Tumor grade						
	Well differentiated		Moderately differentiated		Poorly differentiated		
Laryngeal SCC	**N**	**%**	**N**	**%**	**N**	**%**	**P**
NRP1 score							0.012
Negative (0%)	2	16.7	1	5.9	0	0.0	
Score-1 (1-29%)	2	16.7	1	5.9	0	0.0	
Score-2 (30-60%)	6	50.0	8	47.1	3	27.3	
Score-3 (61%+)	2	16.7	7	41.2	8	72.7	
Total	12	100.0	17	100.0	11	100.0	
Median	Score-2		Score-2		Score-3		
Mean rank	14		20.6		27.4		
r=0.477 P=0.002							

Table 6: The difference in median score category of selected NRP1 marker between the 3 tumor grades among cases with Skin SCC.

	Tumor grade						
	Well differentiated		Moderately differentiated		Poorly differentiated		
Skin SCC	**N**	**%**	**N**	**%**	**N**	**%**	**P**
NRP1 score							0.016
Negative (0%)	4	16.7	3	27.3	0	0.0	
Score-1 (1-29%)	6	25.0	0	0.0	0	0.0	
Score-2 (30-60%)	9	37.5	2	18.2	0	0.0	
Score-3 (61%+)	5	20.8	6	54.5	5	100.0	
Total	24	100.0	11	100.0	5	100.0	
Median	Score-2		Score-3		Score-3		
Mean rank	17.2		22.3		32.5		
r=0.422 P=0.007							

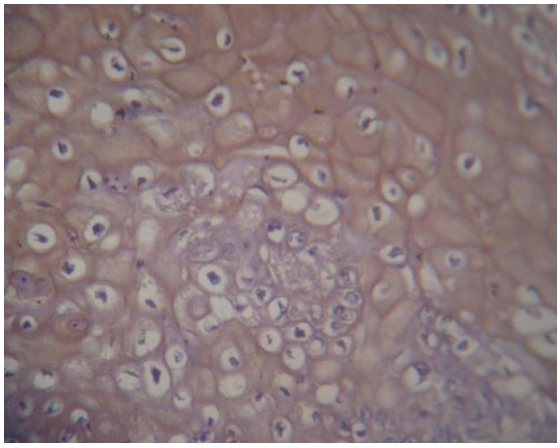

Figure (1): Membranous and cytoplasmic NRP1 expression in OSCC (X40)

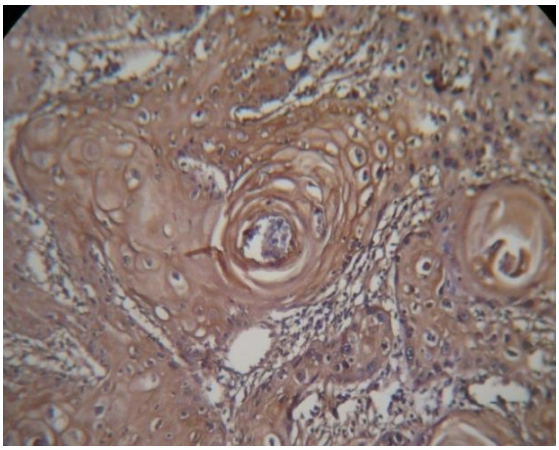

Figure (2):Membranous and cytoplasmic NRP1expression in LSCC (X20)

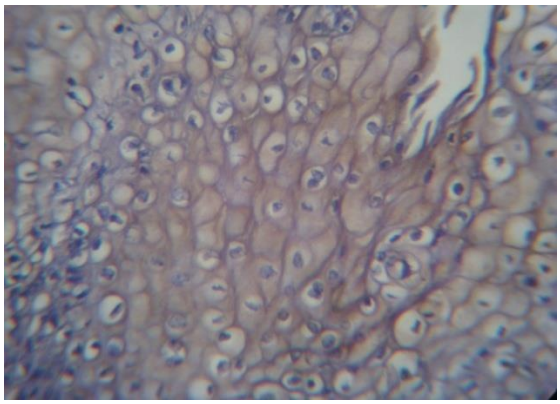

Figure (3): Membranous and cytoplasmic NRP1 expression in Skin SCC (X40)

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الملخص

المقدمة
مستقبل مساعد حديث وليس انزيم اميني حركي وظيفته الرئيسية تحفيز عامل نمو الخلايا المبطنة للأوعية الدموية . أكتشف لأول مرة في الخلايا العصبية (NRP1)يعتبر النيروبلبين 1 المهاجرة. لقد وجد بان هذا المستقبل يظهر عاليا وبنسب متفاوتة حسب نوع السرطان وان ظهوره يعتبر مؤشرا على مراحل متقدمة من المرض. ان الهدف من هذه الدراسة كان للتعرف على تباين ونسب ظهور هذا المستقبل بين ثلاثة انواع من السرطان من اصل واحد ولكن من مواقع مختلفة.
المواد وطرق العمل
تضمنت الدراسة استخدام مائة وعشرون عينة محفوظة بمادة الفورمالين ومطمورة بشمع البارافين تشمل سرطان الخلايا الحرشفية للفم والحنجرة والجلد وبواقع اربعون عينة لكل في جميع الحالات السرطانية. وتم مقارنة النتائج حسب العمر والجنس ودرجة (NRP1)مرض. وحللت الدراسة بطريقة الظهور المناعي النسيجي الكيميائي وباستخدام صبغة ال التمايز النسيجي المرضي.
النتائج
ان نسبة ظهور الصبغة في سرطان الخلايا الحرشفية لكل من الفم والحنجرة والجلد كانت (87.5%), (92.2%) (NRP1)كشف التحليل المناعي النسيجي الكيميائي باستخدام صبغة =P),0.002 (ولكن ظهورها كان على علاقة احصائية قوية مع درجات التمايز النسيجي=P) 0.44(و(82.5%) على التوالي. مع عدم وجود تفاوت كبير في ظهور الصبغة بينهم) على التوالي.=P و (P=0.007)(0.009
الأستنتاج
ترتبط بشكل كبير مع درجة (NRP1) تعتبر جوهرية واساسية في الانواع الثلاثة من سرطان الخلاية الحرشفية . وأن صبغة (angiogenesis)عملية تكون اوعية دموية جديدة التمايز النسيجي للانواع الثلاثة, لذا يمكن ان تعتمد للتكهن بمدى تطور المرض من حيث استفحال السرطان وانتشاره من عدمه في المستقبل.