RESEARCH ARTICLE

Editorial Process: Submission:06/24/2022 Acceptance:10/24/2022

The Correlation Between CD44 and Angiogenesis in Oral Squamous Cell Carcinoma Induced in Buccal Pouch in Syrian Hamster that Underwent Radiotherapy

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Abstract

Background: Angiogenesis with radiotherapy is a significant focus of recent studies to confirm the importance of combined treatments, as vascular control can have a great therapeutic target. Vascular endothelial growth factor is the key mediator of angiogenesis in cancer. In addition, some studies suggest the value of CD44 as a potential early marker of angiogenesis. **Objectives:** Investigating the expression of vascular endothelial growth factor (VEGF) and CD44 in oral squamous cell carcinoma (OSCC) after inducing it in hamsters then undergoing radiotherapy and comparing outcomes before and after therapy to verify changes of these markers. **Materials & methods:** an experimental study consisted of 18 samples of OSCC which induced in right buccal pouch of hamsters (group1) and 18 samples of OSCC which induced in the same way and were exposed to radiation therapy (group2), Biopsies were taken and fixed with formalin, paraffin waxed in conventional H&E and immunostained with monoclonal anti-VEGF and anti-CD44. **Results:** our findings didn't reveal a statistically significant difference in the expression of VEGF($p = 0.342$) and CD44 ($p=0.187$) between group1 & group 2. moreover, we found tumor cells which weren't affected and resistant to radiotherapy, also revealed positive expression of VEGF & CD44, otherwise, we noticed Pearson coefficient was a significant correlation that indicated to a moderate relation. **Conclusion:** cancerous cells that showed a high expression of these markers, give elevated radiosensitivity and resist the treatment. Subsequently, we assure the importance of applying anti-VEGF and/or anti-CD44 as a supportive therapy with radiation therapy.

Keywords: Oral squamous cell carcinoma- angiogenesis vascular endothelial growth factor (VEGF)- radiotherapy- CD44

Asian Pac J Cancer Prev, **23 (10)**, 3571-3576

Introduction

Head and neck squamous cell carcinomas (HNSCCs) is one of the most common cancers worldwide with a prevalence rate about (54%), and it is considered as the 3rd cancer in developing countries, it has the highest mortality rate among head and neck cancers(Ludwig et al., 2019, Ajalyakeen et al., 2020). The 5-years survival rate has improved only marginally in recent decades, which clarifies the need for advancements and researches in diagnosis, prognosis and treatment of head and neck squamous cell carcinoma (C.K. Howlader N et al., 2013.). Moreover, the therapeutic efficacy remains insufficient (Bolandparva et al., 2021).

Angiogenesis defines as the formation of new blood vessels which sprout out of the previous vessels. Growth factors (VEGF, Tumor growth factor TGF-β Angiopoietin, Fibroblast growth factor FGF, and Platelet-derived growth factor PDGF) are a trigger of neovascularization, while cell adhesion molecules also critically control and organize vascular morphogenesis (Adams and Alitalo, 2007). It is common that Tumors induce angiogenesis to provide their oxygen and Nutritional Requirements, and are dependent on an adequate blood supply for repairing and growth, to grow more than 1-2 mm (Carmeliet, 2005). The clinical importance of tumoral angiogenesis as a passive prognostic factor has been demonstrated in many tumors. furthermore, hypo perfusion of tumors resulting hypoxia is assumed to be one of the main reasons for radiotherapy failure (Shintani et al., 2000).

VEGF is considered as a homodimeric glycoprotein with a molecular weight of about 45 kDa, which is the main key of angiogenesis in cancer, in which it is regulator by oncogene expression (Carmeliet, 2005). VEGF-A-targeting monoclonal antibody therapies have provided successful results in diverse types of cancer like colorectal cancer and head and neck cancer (Itashiki et al., 2021). Bevacizumab is a molecularly targeted medication with different Characteristics compared with conventional chemotherapeutic agents. It directly binds to VEGF-A around the tumor and prevents tumor angiogenesis (Yoshida et al., 2018). Moreover, bevacizumab normalizes residual blood vessels and develops the drug delivery system into cancer so that medications reach to cancer at

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a greater concentration. (Itashiki et al., 2021) .

It has long been supposed that angiogenesis inhibitors should not be combined with radiotherapy, because it can increase tumor hypoxia. Therefore, it reduces the effectiveness of radiotherapy (Mazeron et al., 2009). On the other side, Huber et al. studied the association SU11657, an inhibitor of (VEGF, PDGF and C-kit in vitro and in vivo, on a human squamous cell carcinoma cell model delete A431. The triple combination was more effective than each of the modalities used alone or that the combination anti-angiogenic with radiotherapy (Huber et al., 2005). However, most studies show a beneficial effect to combine the two therapeutic modalities, the differences observed in efficacy are difficult to interpret with regard to different irradiation schemes, tumor models, doses and durations of treatment studied. Moreover, the importance of the expression of the inhibited pro-angiogenic factor could influence the result (Gupta et al., 2002).

The Cluster of differentiation 44 (CD44) is considered as a cell surface adhesion receptor that is commonly expressed in many cell types, including epithelial cells, tumor cells and vascular endothelial cells (ECs). The CD44 gene is found on chromosome eleven of the human genome and on chromosome two of the mouse genome. There are two groups of exons in the human and mouse Cd44 genes: exons 1-5 and 16-20 are spliced essentially and presented as standard CD44]CD44s[, while exons 6-15, specified as variable exons versions (1-10), are alternately spliced and inserted between exons 5 and 16, that way encoding the various CD44 isoforms (CD44v) (Chen et al., 2020)

on the other side, the status of CD44 as a main glycoprotein for tumor growth stays unclear. In HNSCC, the CD44 marker is generally referred to as a marker for Cancer Stem Cells (CSCs) (Chen et al., 2018). The CSC hypothesis suggests a small subpopulation of CSCs within the tumor mass of non-CSCs as a highly effective originator of tumor progression, radiochemoresistance and disease recurrence (Prince and Ailles, 2008).

One of studies found a positive correlation of CD44 expression with Micro Vessel Destiny (MVD) in head & neck squamous cell carcinoma. On the other hand, they showed a statistically significant negative correlation in normal oral mucosa (Ludwig et al., 2019).

CD44 deletion in models causes reduced pathological angiogenesis without impacting normal angiogenesis during development, while its overexpression is related to improved tumor angiogenesis, neovascularization, and ischemic angiogenesis (Ludwig et al., 2019)

Material and Methods

This experimental study was applied in Damascus University, between October 2020 and September 2021. Ethical approval for this study was obtained from Damascus University, Ethical committee (decision number 1450, on 18/11/2019).

Animal Housing and Carcinogenesis Induction

36 Four-weeks-old, 80 gr weight male Syrian hamsters were procured and housed in filter-capped polypropylene cages at]23±2°C [and well-kept for a 12 h light/dark cycle in special incubators at Damascus university. Water and nutrition were given frequently. Before beginning the experiment, animals were housed for 2 weeks in a quarantine time. a total number of 36 animals (six weeks old) were divided into 2 groups. Group 1 (n=18) was exposed by painting the right buccal pouch three times per week with a camel-hair brush soaked in 0.5% 7,12-dimethylbenzanthracene (DMBA, Sigma®) dissolved in mineral oil until 14 weeks, then we excised the right buccal pouch figure.1 and prepared samples for incubation within wax blocks. group 2 the same number of animals and same way for carcinogenesis Induction was used (n=18), after 14 weeks of DMBA exposition, the duration for OSCC-induction into buccal pouch (Gupta et al., 2002), then underwent radiation therapy at the Atomic Energy Authority, using a cobalt 60 device, which used for experimental studies and send beams of high-energy rays horizontally Figure.2. Hamsters were exposed till 30 Gy dose cumulatively, where the radiation dose was fractionated into 2 Gy/day per 5 days weekly. were sacrificed (2 models after one week 10 Gy, 2 models after two week 20 Gy, 14 models was sacrificed after the irradiation completely 30 gy). finally, We also excised the right buccal pouch . and prepared the samples for incubation within paraffin blocks.

Sample preparation:
paraffin sections of formalin-fixed tissues were used for both conventional

(H & E) stained sections and Immunohistochemical (IHC) examination. Histological (H and E) stained sections were used for the primary diagnosis and confirmed OSCC-incubation . For immunohistochemical evaluation, 4 µ sections were prepared and loaded on slides. then Slides were deparaffinised and washed in deionized water and treated with antigen retrieval. Peroxide block (3% hydrogen peroxide) was used to block endogenous peroxidase for 12 minutes. After pre-treatment, sections were incubated with primary antibody rabbit monoclonal to VEGF, CD44 antibodies (Bio SB, USA) VEGF(BSB) CD44 (BSB) both of them is ready to use , in a humid chamber at 4°C overnight, performing heat mediated antigen retrieval with citrate buffer at pH 6 before commencing with IHC staining protocol.

IHC scoring

The positive expressions were assessed by the percentage of positive cells in addition staining intensity according to the method described by Rodrigues et la (2018) (Rodrigues et al., 2018) . The percentage of positive cells was rated as follows: 0 (0-5%); 1 (5%-24%); 2 (25%-49%); 3 (5%-74%); or 4 (>75%). whereas Staining intensity was graded as 0 (no staining), 1 (weak), 2 (moderate), and 3 (intense). Finally, the percentage of positive cells and the staining intensity immunostaining score were (added together) to obtain a total score, which ranged from (0 to 7).

Statistical analysis

one-way ANOVA analysis was used to determine if

there was a statistically significant difference between experimental groups, and a Pearson correlation coefficient was used to determine if there a positive or negative correlation.

In our study, the level of significance (P value) was set at 0.05, moreover all statistical analysis including descriptive analysis were done using SPSS (version 23).

Results

Group (1) OSCC-induced without radiotherapy

With H&E staining, all of our samples showed a formation of oral squamous cell carcinoma grade 1 Well differentiated (neoplastic epithelial islands containing with keratin pearls as well as few mitotic figure.ure.s and multinucleated epithelial cells are seen).

IHC

The predominant intracellular distribution for each antigen was: CD44 – membranous only, VEGF-cytoplasmic only.

CD44

Many cancerous cells showed positive expression in all 18 samples; the staining was ranged between moderate and intense (eight cases of OSCC were strongly positive in tumor cells, seven cases expressed moderate positive, three cases expressed weak positive in neoplastic epithelial cells) Figure.3.

VEGF

several of cancerous cells showed positive expression for VEGF, with intense staining in (seven cases of OSCC were strongly positive in tumor cells, nine cases expressed moderate positive, two cases expressed weak positive in neoplastic epithelial cells) Figure 5.

GROUP (2) OSCC-induced through radiotherapy

With H&E we noticed inflammatory reaction and fibrosis in the stroma After radiotherapy

CD44

Many cancerous cells ,which showed radioresistance, had positive expression. Although there was a little of decrease in this expression after 30Gy, but it wasn't statistically significant (P = 0.342) Figure.4 Table.1

VEGF

immunoreactivity was observed in the cytoplasm of the most cancer cells, the results of one-way ANOVA analysis with 95% statistical confidence, we didn't find significant differences of VEGF expression before and through treatment, (percentage and intensity) was not affected by a statistically significant (P = 0.187) in radiotherapy in cells that hadn't any Radiological response after 30 Gy and in whom there was a non-effective.Figure 6 Table.1

Figure 1. Shows Withdrawal Right Buccal Pouch of Hamster before Enucleating

Figure.2. Underwent Radiotherapy by Cobalt 60 Device

Table 1.Association of CD44, VEGF Expression Levels in Induced-Oral Squamous Cell Carcinoma with Group 1,2(a,b,c) (P value according to one way anova's exact test)

	CD44					VEGF				
	median	standard deviation	F value	P value	differantiation	median	standard deviation	F value	P value	differantiation
Induced-OSCC without RT	5.11	1.03	1.707	0.187	No significant difference	95.30	1.60	1.161	0.342	No significant difference
Induced-OSCC after 10 gy (a)	6.00	0.00				5.00	1.41			
Induced-OSCC after 20 gy (b)	5.50	0.71				4.50	0.71			
Induced-OSCC after 30 gy (c)	4.28	0.97				5.64	1.20			

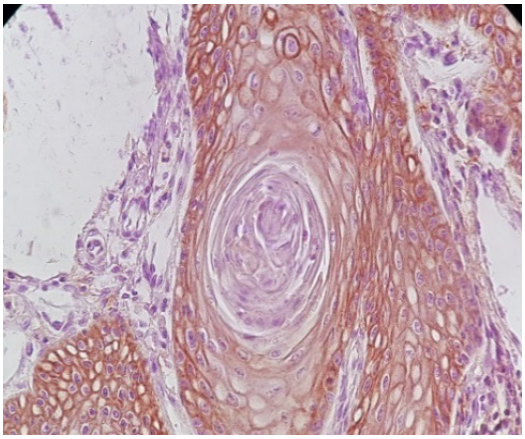

Figure 3. High Positivity for CD44 in the Cell Membrane of Nearly of most Cancerous Cells (group1) Magnification ×400.

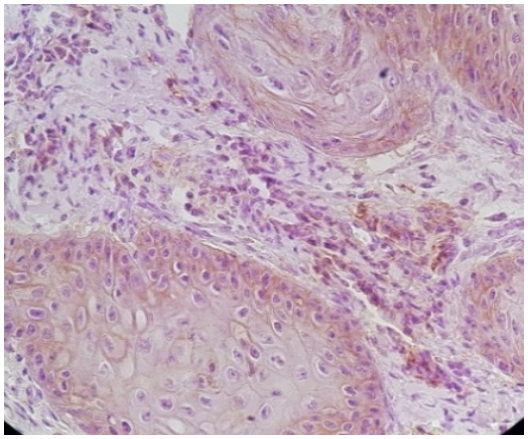

Figure 4. Moderate Positivity for CD44 in the Cell Membrane of some Cancerous Cells (group 2) Magnification ×400.

Table 2. Pearson Correlation Coefficient

	groups	VEGF			Differentiation	relationship side	relationship intensity
		P value	numbers	Correlation value			
CD44	Induced-OSCC without RT (G1)	0.004	18	0.644	Significant difference	POSITIVE	moderate
	Induced-OSCC after 30 gy (G2)	0.024	14	0.597	Significant difference	POSITIVE	moderate

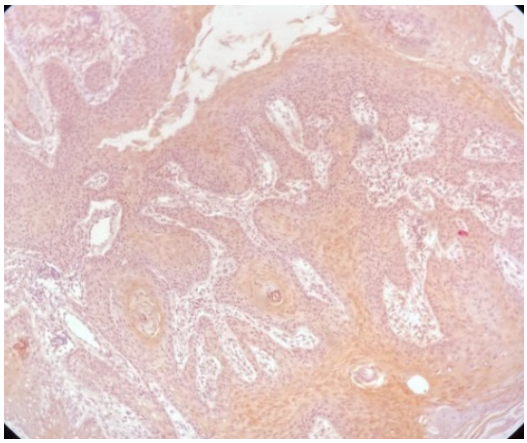

figure 5. Moderate Positivity for VEGF in Cytoplasm of Neoplastic Cells (group1) Magnification ×100.

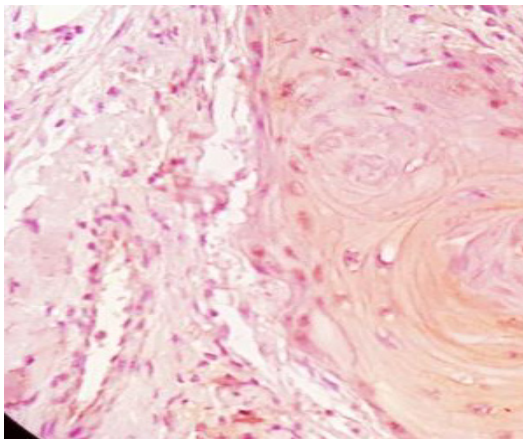

figure 6. Positivity for VEGF in the Cytoplasm of Nearly All Cancerous Cells (group2) Magnification ×400

The relation between CD44 and VEGF in OSCC

Pearson correlation coefficient was significant correlation, that indicated to a modorate relation between the expression of these proteins, and when the expression

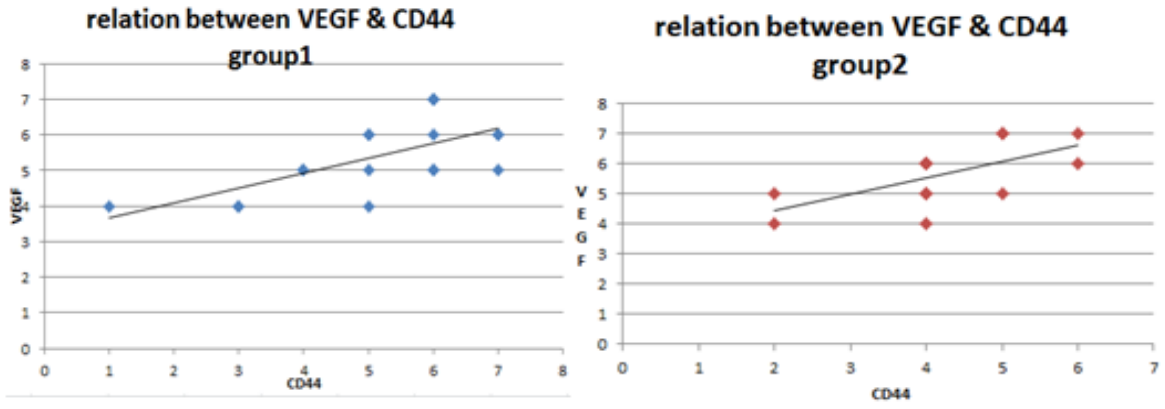

Figure 7. The Relation between the Expression of VEGF and CD44

of one of them increases the other protein expression may increases too. Table 2 Figure.7

Discussion

Head and neck squamous cell carcinomas]HNSCCs[is considered the sixth most

Common cancer in the world. radiotherapy has been the initial treatment for patients affected with an inoperable tumor (Zaid et al., 2016).

Primary tumor growth must be associated with the development of blood vessels. This had led to the development in studying the correlation between tumor angiogenesis and prognosis in cancer patients(Shintani et al., 2000). several studies have reported a relationship between increased tumor vascularity and poor prognosis(Bochner et al., 1995), Since tumor vascularization has of the ultimate importance for tumor improvement, it is supposed that the anti-vascular effect of irradiation may be partly involved in the anti_tumoral effect of radiotherapy (Shintani et al., 2000).

The results of our study are similar to Chitransha et al (Dr Chitransha Srivastava, 2019) who studied VEGF in OSCC with different grades and found the expression of VEGF in neoplastic cells between relatively high to moderate and noticed overexpression with poor-differentiated. That emphasizes a worse prognosis with more cell proliferation with an intense expression. Furthermore, we differed with Chitransha in terms of the correlation of degrees with the expressiveness as we found in this study, some of cases there was an elevated expression in well- differentiated, this difference may due to characteristics of OSCC which is known as heterogeneity

In the present study, we haven't observed a statistically significant (P = 0.187) in VEGF expression of neoplastic cells which underwent radiotherapy that hadn't any Radiological response after 30 Gy and we've noticed some cases had a higher expression compared with group 1 without irradiation. these findings concord with Shintani et al (Shintani et al., 2000) who studied the expression of this marker after undergoing 30 Gy of oral squamous cell carcinoma then have taken a biopsy and found changes in expression before and after treatment Where was found an increase or no change in the expression whereas a few cases with a decrease in the expression, that's also obtained in our study. so thus confirming that cells with VEGF(+) are more resistant to radiotherapy, which leads us to suggest the importance of adding anti-VEGF treatment With radiotherapy, even if it is provided as a preoperative treatment for surgical excision, greatly reduces the possibility of subsequent recurrence. few studies examined CD44 after radiotherapy while we did not find a similar study in OSCC. Nevertheless, our findings are approaching Jong's study (de Jong et al., 2010), who studied CD44 in laryngeal cancer after radiation therapy (RT) and discovered the association of this marker with radiotherapy response in early-stage cancer as well as predicted that cases which had high expression may get a greater possibility of recurrence, they also have emphasized the necessity of more studies to confirm evaluation of these results in different sites of HNSCC.

On the other hand, It was previously reported, that positive expression of CD44 actively interacts with surrounding stromal cells in the tumor microenvironment. The tumor cells incorporate pre-existing vessels and utilize the perivascular niche for their survival (Krishnamurthy et al., 2010). in addition, Some studies observed that positive cells of CD44 are located in the perivascular niche in the models as well as in human tumor tissues. The self-renewal and growth of CD44(+) cells in the perivascular niche is encouraged by factors released by the surrounding endothelial cells, as described previously (Krishnamurthy et al., 2010). therefore, they suggested that the CD44(+) cell subpopulation is enriched in cells with CSC features, which support neovascularization. The migration of endothelial progenitor cells]EPCs[towards the tumor site is promoted by pro_angiogenic factors produced by CSCs (Treps et al., 2017). Cancer stem cells also have the capability to differentiate into endothelial cells and to be incorporated in newly forming vessels (Carmeliet and Jain, 2011). in this present study, we have found a moderate correlation between CD44 and VEGF before and after irradiation so we can suggest targeting these markers as a supportive treatment after radiotherapy to avoid hypoxia which produces from anti-angiogenesis agents.

In conclusion, the results of our present study proved that aberrant expression of VEGF and CD44 in tumor cells didn't appear high radiosensitivity and showed resistance to therapy. Thus, we believe that applying anti-VEGF and anti-CD44 as supportive therapy with radiation therapy should be very useful. On the other hand, The correlation between these two markers may help to find a combined pathway to target radio resistant cells. Hence, be beneficial in reducing tumor progression.

Limitations

In the present study, statistical results were recorded within only grade 1 of oral squamous cell carcinoma. In addition, it was applied to male Syrian hamsters only, to avoid hormonal changes in females.

Author Contribution Statement

The authors confirm contribution to the paper as follows: study conception and design:R.A ; data collection: R.H; analysis and interpretation of results: R.A, A.M; draft paper preparation: R.A. both authors reviewed the results and approved the final version of the manuscript.

Acknowledgements

This manuscript is part of an approved student thesis. We want to assure that Animal Housing were held at faculty of pharmacy, Damascus University and all laboratory tests were held at the Department of oral histology and pathology, Faculty of Dentistry, Damascus University. This work was supported by Damascus University.

Conflict of interest

None.

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