The Correlation of Immunohistochemical Expression of CD20 with the Level of Tumor-Infiltrating Lymphocytes (TILs) and the Histological Grading of Cutaneous Squamous Cell Carcinoma

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***Abstract*- Background:** Tumor infiltrating lymphocytes (TILs) have been important prognostic factor for many malignancies, including cutaneous squamous cell carcinoma (cSCC). TILs are composed of many subsets, categorized into T-cells and B-cells. The tumor immune responses of B-cells are poorly investigated and ignored for years. The role of B-cells in tumor immunology is still controversial due to the antitumorigenic effect and also the ability to increase the tumor progression.
Objective: To analyze the correlation of immunohistochemical expression of CD20 with the level of TILs and histological grading of cSCC.
Methods: This is an analytical research with cross sectional approach using the Hematoxylin Eosin staining for TILs and histological grading evaluation, together with immunohistochemical staining of CD20 for tumor-infiltrating B-cell (TiBC) evaluation. The statistical analysis with Kruskal Wallis method is used to analyze the correlation..
Results: Based on the statistical analysis with Kruskal-Wallis method for 45 samples of patients with cSCC to analyze the correlation of immunohistochemical expression of CD20 with the level of intratumoral TILs and stromal TILs, the p-values are 0,479 and 0,904 ($p>0,005$). The correlation of immunohistochemical expression of CD20 with the histological grading showed the p-value of 0,091 ($p>0,05$).
Conclusion: There are no relation between the immunohistochemical expression of CD20 with the level of intratumoral TILs and stromal TILs at cSCC, but the CD20 expression tends to have correlation with the histological grading of cSCC although it is not significant statistically.
***Index Terms*-:** cutaneous squamous cell carcinoma, TILs, grading, CD20.

I. Introduction

Cutaneous squamous cell carcinoma (cSCC) is the malignant skin tumor derived from the malignant keratinocytes proliferation of the epidermis and the skin appendages, which look similar to the epidermal spinosum layers.[1,2] It is the second most common skin malignancies after the basal cell carcinoma with the approximate incidence rate about 20% of all skin malignancies.[1,3] The etiology and risk factors are including the sun exposure, chemical carcinogen, HPV and HIV infection, chronic skin inflammation, immunosuppresion, and also genetic disorder.[4,5]

There are many factors which influence the prognosis of cSCC. The worse prognosis of cSCC is correlated with many factors, such as the tumor depth more than 2 mm, Clark level invasion of IV/V, perineural invasion, primary mass located on ears or lips, and the poor differentiation of cSCC.[6] Tumor differentiation or histological grading is classified into well differentiation, moderately differentiation, and poor differentiation, based on the degree of anaplasia in the tumor nest, mitotic activity, and the keratin formation.[7]

Tumor-infiltrating lymphocytes (TILs) play an important role in the tumor progression, invasion, metastasis, and also the outcome of the patient. TILs can be differed into intratumoral TILs and stromal TILs. Intratumoral TILs are the lymphocytes which infiltrate into the tumor nests, while the stromal TILs are the lymphocytes found around the tumor nests.[8] A lot of studies had been done to evaluate the role of TILs in solid tumor, such as lung carcinoma, gastric cancer, colorectal carcinoma, hepatocellular carcinoma, breast cancer, ovarian carcinoma, melanoma, and cSCC.[9] Some of them had proven that TILs can be the independent prognostic factor which is more accurate than TNM staging.[10] The previous study about the role of TILs in cSCC has concluded that stromal TILs are closely related to the histological grading of cSCC and has been suggested to be evaluated and reported routinely in the histopathological examination.[11]

TILs has many subsets, such as CD3+, CD4+, CD8+, FoxP3+, αβ Tcells, γδ T cells, B cells, natural killer cells, and natural killer T cells. Each subset plays the different roles in the tumor immunity.[12-14] The role of B cells in the carcinogenesis and tumor immunity is still unclear. Only a few studies had been performed to evaluate its role. Some studies had showed that B cells have the antitumorigenic effect because of their ability to present the tumor antigen and kill the tumor cells directly. Others showed that B cells can inhibit the tumor regression, prevent the cytotoxic response of CD8+ T cells, and finally support the tumor progression.[15-16] The studies about the role of tumor infiltrating B cells (TiBC) in cSCC are still limited. The previous study was performed only to compare the different densities of

CD20 in cSCC patients with renal transplantation and the immunocompetent patients of cSCC. CD20 is a membrane-embedded surface molecule which is commonly used to evaluate B-cells. CD20 is expressed on every stage of B cell differentiation, except the plasma cells.[17-18] This study is aimed to evaluate the correlation between the immunohistochemical expression of CD20 with the level ot TILs and histological grading of cSCC, as B cells, expressed by CD20 is part of the TILs.

II. MATERIAL AND PRODUCT

This is an analytical study with cross sectional approach, conducted at the Anatomical Pathology Laboratory of Faculty of Medicine, Universitas Sumatera Utara and also at the Anatomical Pathology Unit of Haji Adam Malik Medan General Hospital. This research was held from July 2019 until June 2020, after approved by Universitas Sumatera Utara and Haji Adam Malik General Hospital Health Research Ethics Committee.

The sample size needed for this research was calculated based on the the minimum sample size formula and at least 43 samples were needed. We used 45 samples from the slide and the paraffine blocks of the patients diagnosed as cSCC. The inclusion criteria for this research were patients' slides or paraffine blocks with a pathological diagnosis of cutaneous squamous cell carcinoma which are adequate and representative with epidermal and dermal structures. The exclusion criteria included the following: (1) Slides or paraffine blocks contained minimal tissues that are not possible to be cut again for immunohistochemical staining; (2) Slides only showed the tumor cells with minimal stromal area for evaluation of TILs.

Histological grading and the level of TILs infiltration were evaluated independently by researcher, together with two pathologist using Olympus CX23 microscope. The histological grading of the cutaneous squamous cell carcinoma is based on the degree of differentiation and keratinization and divided into: (1) Well differentiated cSCC, characterized by squamous epithelium that is easily recognisable with abundant of keratinization. The epithelium is obviously derived from squamous epithelium and intercellular bridges (prickles) are readily apparent. The tumors display minimal pleomorphism and mitotic figures are mainly basally located; (2) Moderately differentiated cSCC falls in between the well differentiation and poor differentiation. The epithelial structures are more disorganized in which the squamous epithelial derivation is less obvious. Nuclear and cytoplasmic pleomorphism are more pronounced. The mitotic figures, including the atypical mitosis, can be found more easily. Tumors show less keratinization, often being limited to the formation of keratin pearls; (3) Poorly differentiated cSCC, is more difficult to establish the true nature of the lesion, unless intercellular bridges or small foci of keratinization are found. Tumors showed highly atipia nuclei with lots of mitotic figures.[19-20]

The level of TILs infiltration was determined after evaluate all the fields. TILs infiltration was divided into intratumoral TILs and stromal TILs. Intratumoral TILs are lymphocytes which infiltrated into the tumor nests and were in contact with the tumor cells, while stromal TILs are lymphocytes which infiltrated surrounding the tumor nests. TILs infiltration were categorized into four level: (1) Score 0 for no infiltration of lymphocytes; (2) Score 1 for minimal lymphocytes infiltration which were less than 10 cells / HPF; (3) Score 2 for moderate lymphocytes infiltration in which lymphocytes could be seen easily, but not in large aggregates; (4) Score 3 for massive infiltration in which the infiltration the lymphocytes formed large aggregates and could be found in 50% tumor area.[21]

Paraffine blocks were also cut once for immunohistochemical staining. Immunostaining was performed by using CD20 (L26) monoclonal mouse anti-human clone ready-to-use from Leica. Positive control was human tonsil. The CD20 expression was observed at the cell membrane. The density of the CD20 expression was categorized into three level: (1) Score 0 for no discernible infiltrates; (2) Score 1 for scattered cells without dense infiltrates; (3) Score 2 for dense infiltrates with formation of tertiery lymphatic structures (TLS).[22]

III. RESULT

From 45 samples of cSCC which were evaluated ini this study, 19 cases (42,22%) were well differentiated tumors, 16 cases (35.56%) were moderately differentiated tumors, and 10 cases (22.22%) were poorly differentiated tumors. Evaluation for intratumoral infiltration of TILs showed that 13 cases (28.88%) had minimal infiltration of intratumoral TILs, 25 cases (55.56%) had moderate infiltration of intratumoral TILs, and 7 cases (15.56%) had massive infiltration of intratumoral TILs. Evaluation for stromal infiltration of TILs showed that only 3 cases (6.67%) had minimal infiltration of stromal TILs, 16 cases (35.55%) had moderate infiltration of stromal TILs, and 26 cases (57.78%) had massive infiltration of stromal TILs. The distribution samples for immunohistochemical staining of CD20 showed that 10 cases (22.22%) had no discernible infiltration of B cells (no expression of CD20), 29 cases (64.45%) had scattered B cells without dense infiltrates, and 6 cases (13.33%) had dense infiltrates of B cells with the formation of TLS.

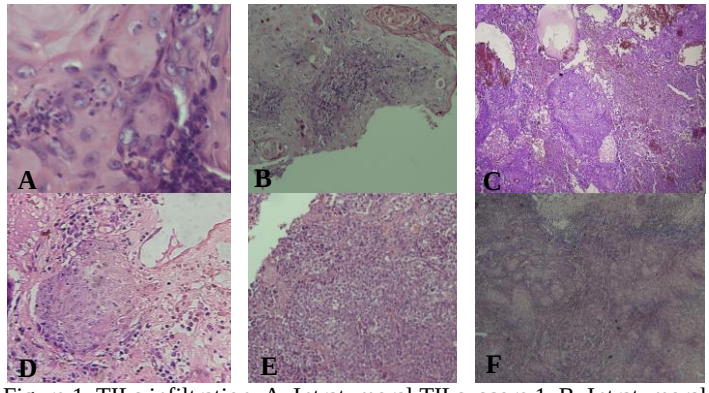

Figure 1. TILs infiltration. A. Intratumoral TILs, score 1. B. Intratumoral TILs, score 2. C. Intratumoral TILs, score 3. D. stromal TILs, score 1. E. Stromal TILs, score 2. F. Intratumoral TILs, score 3.

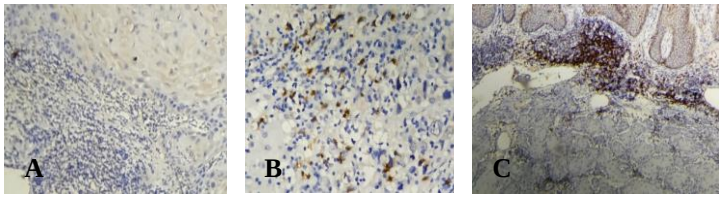

Figure 2. Imunohistochemical expression of CD20. A. Score 0 (no discernible infiltrates) B. Score 1 (scattered cells without dense infiltrates. C. Score 2 (dense infiltrates with formation of TLS)

Statistical analysis with Kruskal-Wallis method showed that no statistical correlation was found between the immunohistochemical expression of CD20 with intratumoral TILs infiltration (p-value = 0.479) and stromal TILs infiltration (p-value = 0.904) at cSCC. There is a tendency of correlation between the immunohistochemical expression of CD20 and histological grading of cSCC, although statistical analysis was not significant enough with the p-value of 0.091 (p-value > 0.005). The data are available in table 2, table, 3 and table 4 (see below).

IV. DISCUSSION

Histological grading has been one of the important prognostic factors for many solid malignant tumors, including cSCC. Lots of previous studies had been conducted to evaluate the role of immnune response for the tumor development and progression. It had been proven that tumor-infiltrating lymphocytes (TILs) can be one of the important factors that impact the prognosis of malignancy.[10] Stromal infiltration of TILs are evaluated more commonly in many malignant tumors, such as breast cancer, squamous cell carcinoma of the head and neck, ovarian cancer, prostatic adenocarcinoma, etc.[9] A recommendation had been proposed to evaluate the TILs infiltration in breast cancer by the International TILs Working Group and stromal TILs are suggested to be evaluated for this purpose.[23] In the previous study about TILs in cSCC, stromal TILs had been proven to have a strong correlation with the histological grading of cSCC.[11]

TILs are actually composed of many subsets, such as CD4+, CD8+, FoxP3+T cells, αβ T cells, γδ T cells, B cells, natural killer cells, and natural killer T cells.[12-13] Each subset plays its role in the tumor immunity response. Although B cells are not the major component of TILs, B cells are believed to have a significant role in the immune response because of their ability in producing the humoral antibodies, facilitating the response of T cells, presenting the antigen, and also their direct cytotoxity capabilities against the tumor cells.[17,24] Studies about the tumor infiltrating B cells (TiBC) had been done for malignancies in many organs, such as the non-small cell lung carcinoma, prostatic adenocarcinoma, ovarian cancer, colorectal carcinoma, uterine cervix cancer, and also breast cancer.[16] Garaud, *et al*. had concluded that the higher density of B cells infiltration in the triple negative breast cancer and HER-2 enriched breast cancer has a correlation with the better outcomes.[25] Mullins, *et al*. did not evaluate the density level of B cells infiltration in colorectal carcinoma, but they focused to study about the ability of B cells in presenting the tumor antigen and secreting the immunoglobulin A, G, and M.[26]

B cells, together with the T cells, will form the TILs aggregation which is best known as tertiery lymphoid structures (TLS). B cells can be evaluated from their surface molecule expression and many studies had used CD20 because CD20 is expressed in every stage of B cell differentiation, except the plasma cells.[17-18] The correlation of the immunohistochemical expression of CD20 and the TILs infiltration, including the intratumoral and stromal infiltration, did not show the statistical correlation based on the Kruskal-Wallis statistical analysis method. Probably, this is due to the composition of cutaneous immune cells types and their ways of migration. CD8+ T cells are more commonly found at the epidermis, while the CD4+ T cells are found mostly at the dermis. The CD4+ T cells and CD8+ T cells will circulate continuously between the skin and the draining lymph node and play an important role in detecting the tumor cells. The migration of the CD8+ T cells are mostly determined by CCL27, a chemokine produced by the keratinocytes. The homing of T cells at the skin is generally determined by the memory cells which express CD45RO+. The memory cells which express these surface molecules are the memory T cell. This indicates that the migration pattern and homing of T cells at the skin are determined by the previous antigen at the same tissue.[27] The presentation of tumor antigen to the T cell lymphocytes can be conducted by the dendritic cells and also the B cells. The dendritic cells only initiate the immune response, while the B cells can give persistent stimulation and can recognize the specific antigen even in the low concentration of the antigen, therefore the antigen can be presented to the T cell persistently and the anergy of T cells can be avoided.[28] B cells also induce the proliferation of T cell. However, normally, the B cells are only found in a very small proportion at the skin and also at TILs component.[17,29] The migration of the B cells to the skin includes many important stages. After produced at the bone marrow, B cells will migrate to the secondary lymphoid organs, such as spleen, peripheral lymph nodes, lymphoid tissues of the gastrointestinal tract and also the mesenteric lymph nodes. At the secondary lymphoid organs, the maturation and differentiation of B cells to become the immunocompetent of effector cells are occurred. Then the mature B cells will circulate to the peripheral tissues. The migration is controlled by a lot of interaction between the unique combination of specific adhesion molecules (selectin and integrin) with the chemokines and their receptors. Nevertheless, these processes are particularly occurred at the gastrointestinal tracts, while the mechanism of migration and recruitment of B cells to the skin have yet been understood. Recent finding showed that the chemokines for homing system of T cells are also identified at the B cells. The exact signaling for the last stage of the lymphocyte trafficking involves the transmigration of cells across the vascular endothelia, which may include ICAM-1, vascular cell adhesion molecule (VCAM)-1, and CD47, but they are still being investigated.[29]

The formation of tertiery lymphoid structure (TLS), which had been categorized as score 3 of TILs infiltration in this study actually involves many types of cells, such as lymphoid tissue-inducer cells, stromal cells, dendritic cells, B cells, and also the subpopulation of T cells.[30-31] In the pathological condition, the reactive stromal cells will produce signaling matrix to induce the specific tissue to overexpress lots of inflammatory mediators, such as CCL21 and CXCL13 and also recruit the leucocyte to the

local lesion to form the TLS. TLS is a tertiery lymphoid structure, formed by the lymphocyte aggregation which is located ectopically at non-lymphoid organs. TLS contain of B cells, dendiritc cells, CD4+ T cells, and also CD8+ T cells. TLS plays an important role in the recruitment, activation and proliferation of B cells and T cells.[31-32] Recent studies had shown that TLS increase the lymphocytes recruitment by expressing CXCL10, CXCL12, CXCL13, CCL19, and CCL21. Transforming growth factor-β can increase the ability of CD8+ T cells at the peripheral circulation to recruit B cells to the tumor mass by expressing CD103 and secreting CXCL13. Inhibition of TGF-β will stop the production of CXCL13 and downregulate the recruitment of B cells, These indicate that the activation of CXCL13, CD103, and CD8+ T cells are closely related to the recruitment of B cells and the TLS formation.[31]

The histological grading of cSCC is based on the degree of anaplasia in the tumor nests, mitotic activities, and the keratin formation. Tumors with high proliferation usually show higher histological grading.[7] Control of tumor development is affected by the the ability of immune respon to recognize and eliminate the tumor cells. Interaction of various immune cells at the tumor microenvironment can cause the antitumorigenic or protumorigenic effects.[12-13] Statistical analysis with Kruskal-Wallis method to evaluate the correlation of immunohistochemical expression of CD20 with the histological grading of cSCC showed that there was a tendency of correlation between them, although it was not statistically significant. This is probably due to the dual role of B cells in tumor immunity, to inhibit the tumor progression and also tu increase the proliferation of tumor cells.[31,33]

Inhibition of tumor growth is related with the ability of tumor-infiltrating B cells (TiBC) in producing antibodies, forming the antigen-antibody complex, presenting the tumor antigen, and secreting the cytokines in order to eradicate the tumor cells or to influence the function of immune cells in the microenvironment of tumor. If the tumor growth can be inhibited, then the diffentiation of tumor will be better.[31,34]

Production of antibodies is the important way of B cells to show the antitumor immune response. Studies had concluded that B cells could neutralize the antibodies, prepare antibodies, and form the antibody-antigen complex. A research showed that TiBC can be activated by MCA205 tumor cells. After being activated, TiBC will produce IgG2b antibodies which will specifically bind the tumor cells and produce the antitumorigenic effect. The research also indicated that TiBC would eradicate the tumor cells which were wrapped in the IgG through the antibody-dependent cell-mediated cytotoxity or dissolve the tumor cells by the antibody-mediated activation of the complement system.[31]

Activated B cells also have main function as antigen presenting cells (APC). Various studies had shown that costimulatory molecules, chemokines, and adhesion molecules at the surface of B cells are highly expressed under the influence of antigen receptors, lipopolysaccharides, and CD40 ligand, thus increase the ability of B cells as APC. Activated B cells by CD40 ligand can express chemokines and costimulatory factors, such as CCL2, CXCR4, CCL5, CXCL5, and CXCL10 and induce antigen-specific CD8+ T cells and CD4+ T cells to produce antitumor immune response. TiBC also present antigen even in the low concentration of antigen for a long period. This is very important for the persistency and proliferation of tumor-infiltrating T cells and the antitumor immune effects.[31] Tumor-infiltrating T cells consist of many subsets. At the epidermis, the most common T cells are CD8+ T cells which have a strong relationship with the better prognosis and survival for patients with malignant tumors. Besides that, the high level of CD8+ T cells also can be found at the tumor with less aggresivity or tumor with better differentiation.[8,27]

B cells also secrete many cytokines to regulate the tumor immunity. Be-1 cells, which are initiated by T helper1 cells (Th1 cells) and antigen, will produce the cytokines, such as IFN-γ, TNF-α, and IL-12. The Be-2 cells, which are initiated by Th2 cells, will produce IL-2, IL-13, TNF-α, IL-6, and IL-4.[31,35] These cytokines play important roles in antitumor immune responses. Studies had shown that IL-12 will induce the production of various other cytokines, such as IFN-1 and also increase the proliferation and antitumorigenic effects of T lymphocytes and NK cells. TiBC also interact with the microenvironment of tumors and play their roles. The most important interaction in tumor immunity can be seen in the interaction of B cells and T cell, which can form the TLS. The density of TiBC (evaluated from the immunohistochemical expression of CD20) is also related with the expression of granzyme B and IFN-1, which are the markers for activation of the cytotoxicity of T cells and NK cells. Therefore, the density of TiBC is actually related with the activation of CD8+ T cells and CD56+ NK cells in the microenvironment of tumor.[31,36]

B cells also have negative effects for antitumor immune response. The B reg cells, a subset of the TiBC, play important roles in the development and progression of tumor growth.[31,33,36] Tumor cells and IL-21 induced granzyme B expressing B reg cells will interact with the tumor cells and increase the ability of immune escape of the tumor cells. B reg will produce TGF-β, which will influence the epithelial-mesenchymal transition (EMT) at the tumor tissue.[31,37] Besides the TGF-β, B-reg cells also produce IL-10 and IL-35. Breg secreting IL-10, also known as B10, will increase the changes of Th0 cells to Th1 cells or Th2 cells, thus inhibit the proliferation and activation of T cells. Moreover, there are also increases of expression for FoxP3 and CTLA4 of T reg cells, which act as the tumor suppressor marker. B reg cells are also able to increase the transformation of CD4+ T cells to FoxP3 T reg and T reg secreting IL-10. Studies had shown that the loss of IL-35 secretion by B reg and T reg cells can increase the activation of macrophage and T cells, and also increase the ability of B cells as APC. B reg cells can also dismiss the toxic effect of NK cells by secreting IL-10. The main transcription factor of IL-10 is STAT3. By the activation of STAT3, B reg cells can inhibit the tumor immunity response and fasten the tumor growth.[31]

This was a retrospective study with some limitation. The samples used in this study derived from the tumor biopsy and tumor mass excision. Although the specimen contained enough tumor cells and stroma for evaluation of TILs, the presence of B cell in normal skin should be considered. Normally, B cells can be found in the dermal layer which probably cannot be obtained optimally from tumor biopsy. The density of TiBC itself is correlated with the activation of CD8+ T cells and CD56+ NK cells at the microenvironment of tumor. The interaction between the CD8+ T cells and the B cells had been proven to increase a

better outcome for some malignant tumors. It should be evaluate too in cSCC. Besides that, this study did not evaluate the the protumorigenic effect of the specific subset of B cells. These limitations will give further consideration in future studies.

V. CONCLUSION

After conducted this study, we conclude some points in the following:

1. The biopsy specimen of cSCC should reach the midreticular dermis for the better evaluation of tumor-infiltrating lymphocytes, especially for the B cells.
2. There is no statistical correlation between the immunohistochemical expression of CD20 with the level of intratumoral and stromal TILs at cSCC.
3. CD20 expression tends to have correlation with the histological grading of cSCC although it is not significant statistically.

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Table 1. Distribution of cutaneous squamous cell carcinoma based on the histological grading, level of tumor-infiltrating lymphocytes, and the immunohistochemical expression of CD20

Variable	Total (n)	Percentage (%)
Histological Grading		
• Well differentiated	19	42.2
• Moderately differentiated	16	35.56
• Poorly differentiated	10	22.22
Level of intratumoral TILs		
• None	0	0
• Minimal infiltration	13	28.88
• Moderate infiltration	25	55.56
• Massive infiltration	7	15.56
Level of stromal TILs		
• None	0	0
• Minimal infiltration	3	6.67
• Moderate infiltration	16	35.55
• Massive infiltration	26	57.78
Immunohistochemical expression of CD20		
• None	10	22.22
• Scattered cells without dense infiltrates	29	64.45
• Dense infiltration with tertiery lymphoid formation (TLS)	6	13.33
Total	45	

Table 2. Correlation of immunohistochemical expression of CD20 and the level of intratumoral TILs

Variable	Level of Intratumoral TILs								Total	p-value
	Score 0		Score 1		Score 2		Score 3			
	n	%	N	%	N	%	N	%		
Immunohistochemical expression of CD20										
• No expression	0	0	3	30	7	70	0	0	10	
• Scattered cell without dense infiltration	0	0	8	27.7	17	58.6	4	13.8	29	0,479
• Dense infiltration with formation of TLS	0	0	2	33.3	1	16.7	3	50.0	6	
Total	-	-	13		25		7		45	

Table 3. Correlation of immunohistochemical expression of CD20 and the level of stromal TILs

Variable	Level of Stromal TILs								Total	p-value
	Score 0		Score 1		Score 2		Score 3			
	n	%	N	%	N	%	n	%		
Immunohistochemical expression of CD20										
• No expression	0	0	0	0	4	40	6	60.0	10	
• Scattered cell without dense infiltration	0	0	2	6.9	11	37.9	16	55.2	29	0.904
• Dense infiltration with formation of TLS	0	0	1	16.7	1	16.7	4	66.6	6	
Total	-	-	3		16		26		45	

Table 4. Correlation of immunohistochemical expression of CD20 and the histological grading of cutaneous squamous cell carcinoma.

Variable	Histological Grading						Total	p-value
	Well differentiated		Moderately differentiated		Poorly differentiated			
	N	%	n	%	N	%		
Immunohistochemical expression of CD20								
• No expression	6	60	4	40	0	0	10	
• Scattered cell without dense infiltration	10	34.5	9	31.0	10	34.5	29	0.091
• Dense infiltration with formation of TLS	3	50.0	3	50.0	0	0	6	
Total	19	-	16		16		45	