Immunohistochemical Expression of CD44 and ALDH1 in Oral Submucous Fibrosis and Oral Squamous Cell Carcinoma

Khin Soe[1], Moe Thida Htwe[2] and Zaw Moe Thein[3]

[1]Lecturer, Department of Oral Medicine, University of Dental Medicine, Mandalay.

[2]Associate Professor, Department of Oral Medicine, University of Dental Medicine, Mandalay.

[3]Professor & Head, Department of Oral Medicine, University of Dental Medicine, Yangon.

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Abstract

Background: In potentially malignant disorders and oral squamous cell carcinomas (OSCC), hyaluronan receptor cluster of differentiation 44 (CD44) and aldehyde dehydrogenase 1 (ALDH1) were often used as cancer stem cell (CSC) markers. The aim of the present study was to identify and compare CD44 and ALDH1 protein markers in relation with pathological variance of OSMF and OSCC.

Materials and method: Thirty specimens of OSMF and 34 specimens of OSCC patients with primary OSCC, from the Department of Oral Medicine, University of Dental Medicine, Yangon, were analyzed cross-sectional study for the expression of CD44 and ALDH1.

Result: CD44 was expressed in 80% of OSMF and 58.82% of OSCC samples while ALDH1 was expressed in 56.67% of OSMF and 55.88% of OSCC samples. CD44 and ALDH1 expression patterns were not completely overlapping within OSMF and OSCC cases. Co-expression of CD44 and ALDH1 in OSMF was 56.67% while 41.18% in OSCC. Kruskal-Wallis test for CD44 and ALDH1 in OSMF was 8.980 ($p = .030$) and 12.565 ($p = .028$) while Kruskal-Wallis test for CD44 and ALDH1 in OSCC was 8.304 ($p = .081$) and 5.900 ($p = .207$). CD44 and ALDH1 showed lesser expression in association with lymphoplasmacytic infiltration at the epithelial-connective tissue junction of OSMF. Correlation of CD44+/ALDH1+ cells and lymphoplasmacytic infiltration in OSCC was statistically significant ($p = .026$).

Conclusion: In conclusion, CD44 and ALDH1 expression showed a more distinct distribution pattern in potentially malignant lesion, OSMF. However, these markers were not sufficient to precisely isolate the CSC subpopulation from

the tumor bulk. Further protein marker was needed to precisely define the CSC subpopulations in OSMF and OSCC. The finding of ALDH1- and CD44-positive cells in epithelium of OSMF and in adjacent non-tumor epithelium as well as tumor portion of OSCC suggests that changes were already underway, as these enzymes tend to be present in cells with a high tumorigenic potential. Tumor-host immune reaction took part an important role in expression of these markers in oral squamous cell carcinoma.

Introduction

Most oral squamous cell carcinomas are preceded by clinical premalignant lesions and conditions like oral leukoplakia, erythroplakia, and oral submucous fibrosis (OSMF).[1] OSMF, now globally accepted as an Asian disease, has one of the highest rates of malignant transformation amongst potentially malignant oral lesions and conditions.[2] The reported malignant transformation rate of OSMF to oral squamous cell carcinoma (OSCC) is 7–13% with a long-term follow-up study recording an annual malignant transformation rate of 0.5%. [3]

Cancer stem cellis defined as a cell within a tumor that possesses the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumor.[4] It is important to note that this defines CSC functionally as the cancer cell with stem cell-like properties including self-renewal and pluripotency. These two essential properties provide cancer with long-lasting CSC to give rise to heterogeneous progenies to maintain the tumor or recapitulate the tumor elsewhere (metastasis) or after treatment (relapse).

CD44 is a cell surface glycoprotein receptor for hyaluronic acid (HA), and it seems to be involved in cell adhesion, migration, and metastasis of cancer cells.[5]

The aldehyde dehydrogenase (ALDH) family of enzymes is comprised of cytosolic isoenzymes that oxidize intracellular aldehydes and contribute to the oxidation of retinol to retinoic acid in early stem cell differentiation.[6] High ALDH1 activity has been used to isolate normal hematopoietic and central nervous system stem cells.[7]

It could be hypothesized that CD44 and ALDH1 might be related with malignant transformation of OSMF and invasiveness of OSCC. Purpose of this study was to identify expression of these markers in OSMF and OSCC, and compare with pathological grades.

Materials and method

Thirty-four OSCC specimens and thirty OSMFs specimens in paraffin-embedded tissue sections were provided through the Oral Pathology Department, University of Dental Medicine, Yangon. The human subject protocol was approved by the Institutional Ethical Board according to the NIH guidelines. Hospital based

cross-sectional descriptional study was used. All specimens provided by the Oral Pathology Department were recoded. In these specimens, OSCC differentiations were determined by Oral Pathologist as well, moderate and poorly differentiation. Histopathologic grading of OSMF was subdivided into (1) very early, (2) early, (3) moderately advanced, and (4) advanced, according to the Pindborg and Sirsat (1966) classification.[8] The OSCC cases were graded as Bryne's Invasive Tumor Front classification.[9] These cases of OSMF and OSCC along with normal oral mucosa were studied for IHC expression of CD44 and ALDH1.As for control study, fifteen cases of oral mucosal lesions of patients who came to UDM for surgery apart from OSMFs and OSCCs without tobacco and alcohol habits were chosen.

Paraffin-embedded sections were deparaffinized with xylene and a series of concentrations of ethanol, blocked with 20% IgG, and stained with ALDH1 from Santa Cruz Biotechnologies (Santa Cruz, CA) and mouse anti-human CD44 from (MiltenyiBiotec Inc. Aubum, CA). Sections were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) in mounting medium (from Vector Laboratories, Inc. Burlingame, CA) demonstrating nuclei. Negative controls were stained without primary antibodies. Stained sections were examined under microscope. Positive rates for CD44 and ALDH1 were counted in all OSCCs and OSMFs sections.

Data analysis

Data analysis was done by using SPSS version 19, IBM product. Quantitative data was expressed as means±SD, and the difference in positive rates of CD44 and ALDH1 in OSMF specimens or OSCC specimens were analyzed by using Kruskal-Wallis and Chi-square test. A p-value of less than 0.05 was considered as statistically significant.

Results

General expression of CD44 and ALDH1 in OSMF case

The majority of OSMF cases (24/30; 80%) expressed a minimum of one protein (Table 6). Only six samples (20%) expressed neither CD44 nor ALDH1. CD44 expression was detected in 80% (24/30) of studied OSMF cases. In the CD44+ OSMF cases, 83.33% (20/24) showed extensive area of protein expression, two cases (8.33%) revealed diffused area of protein expression and the remaining two cases (8.33%) was seen localized area of protein expression. CD44 expression intensity was scaled within OSMF cells. Sixty % (18/30) of the CD44+ OSMF expression for the receptor was strong (+++), 13.33% (4/30) the CD44 expression was moderate (++) and 6.67% (2/30) of samples the CD44 level was weak (+). Epithelial cells were solely CD44+ or CD44 expressing epithelial cells were located in the oral lining epithelium.

ALDH1 expression was detected in 56.67%

(17/30) of the studied OSMF samples (Table 6). In the ALDH1+ OSMF cases, 23.53% (4/17) showed extensive area of protein expression and only one case (5.88%) was detected diffused area of expression. Majority of ALDH1+ OSMF cases (12/17; 70.59%) revealed localized area of protein expression. None of the ALDH1+ OSMFs this enzyme was expressed strongly in the majority of epithelial cells. Five cases (16.67%) were expressed moderate intensity and majority of ALDH1+ OMFS cases (12/30; 40%) was evident with mild intensity of protein expression. ALDH1 expressing cells were found to be singular or in groups. More often ALDH1+ OSMF cell groups were located deeper areas of connective tissues.

More than half of the analyzed OSMFs (17/30; 56.67%) expressed co-staining of CD44 and ALDH1 (Table 6). In these OSMF cases the expression pattern of CD44 and ALDH1 intersected, but did not completely overlap. In CD44+/ALDH1+ OSMF cases, CD44 showed mainly extensive area expression (14/17; 82.35%) and ALDH1 showed localized area expression (12/17; 70.59%). High staining intensity of CD44 was seen in 60% of OSMF cases and ALDH1 was detected 56.67% of OSMF cases with mild and moderate intensity.

General expression of CD44 and ALDH1 in OSCC cases

The majority of OSCC cases (25/34; 73.53%) expressed a minimum of one protein (Table 7). Percentage of CD44+ OSCC and ALDH1+ OSCC was nearly the same, 20/34 (58.82%) and 19/34 (55.88%) respectively. Only nine samples (26.47%) expressed neither CD44 nor ALDH1. CD44+ OSCC cases showed extensive area as well as localized area of expression nearly the same amount cases, 8/20 (40%) was for the former and 9/20 (45%) was detected as localized expression. Three cases (15%) showed diffused expression. CD44 expression intensity was scaled within OSCC cells (Table 9). In OSCC, 23.53% (8/34) showed CD44 expression for the receptor was strong (+++), 2.94% (1/34) was moderate (++) and 32.35% (11/34) of samples the CD44 level was weak (+).

ALDH1 expression was detected in 55.88% (19/34) of the studied OSCC samples (Table 7). In the ALDH1+ OSCC cases, seven cases (36.84%) showed diffused area of expression and 12 cases (63.16%) revealed localized area. Staining intensity for ALDH1 was slightly faint. ALDH1 expressing cells were found to be singular, in groups or throughout the entirely cell nest. Staining intensity of both markers in OSCC cases was frequently the same.

CD44+/ALDH1+ OSCC cases are detected in 41.18% (14/34) of studied OSCC samples (Table 7). In these cases, the expression pattern of CD44 and ALDH1 intersected, but did not completely overlap. In CD44+/ALDH1+OSCC 14 cases, CD44 showed (7/14) 50% extensive area expression while ALDH1 was detected localized area expression eight cases (57.14%).

Expression pattern of CD44 and ALDH1 in OSMF and OSCC

The results of present study showed positive CD44 and ALDH1 expression in both basal and supra-basal cells, which may indicate that the immunohistochemical technique here employed was not appropriate to identify cancer stem cells in HNSCCs based on these markers, as also reported by other authors. Moreover, no specific markers have been established for the identification of cancer stem cell subpopulations in HNSCCs, as is the case with other tumors. Notwithstanding, immunohistochemistry did allow to identify the location of positive cells in different tumor areas (tumor center and invasive front), as well as in the different layers of adjacent non-tumor epithelium and basal and para-basal cell layers of OSMF.

Immunostaining results obtained for the OSCC and non-tumor epithelium from OSMF showed that tumors with ALDH1+ and CD44+ cells also presented positive surrounding epithelial tissues and non-tumor epithelium of OSMF. This followed the concept of field cancerization, according to which carcinogen-induced changes would be present in HNSCC-related non-tumor tissues before morphological alterations can be found. The finding of ALDH1+ and CD44+ cells in epithelium of OSMF and in adjacent non-tumor epithelium as well as tumor portion of OSCC suggests that changes were already underway, as these enzymes tend to be present in cells with a high tumorigenic potential.

In the present study, general expression of CD44 was 80% in OSMF and 58.82% in OSCC, while general expression of ALDH1 was 56.67% in OSMF and 55.88% in OSCC. In control cases, these markers were seen only 6.67%. Co-expression of CD44 and ALDH1 was 56.67% in OSMF, 41.18% in OSCC and 0% in control cases. Chi-square test for CD44 and ALDH1 was 37.600 (p= .000) and 14.000 (p= .003) in OSMF, 15.412 (p= .004) and 16.000 (p= .003) in OSCC and CD44+/ALDH1+ in OSMF was .533 (p= .465) and in OSCC 1.059 (p= .303), respectively.

ALDH1 and CD44 immunostaining results were higher in non-tumor epithelium of OSMF, suggesting that these markers could be used as an instrument to predict higher or lower risk of malignant transformation. Moreover, in the non-tumor epithelial portions of OSMF, CD44 and ALDH1 was seen in 80% and 56.67%; in tumor portion and non-tumor portion of OSCC, CD44 and ALDH1 was seen in 58.82% and 55.88% respectively. This finding indicates the presence of cells with a highly tumorigenic potential even in cancer-free zones.

Correlation of markers with lymphocytes in epithelial-connective tissue junction of OSMF and lymphoplasmacytic infiltrations around the tumor cells of OSCC.

CD44 is an adhesion protein expressed on

inflammatory and vascular cells. CD44 supports the adhesion of activated lymphocytes to endothelium and smooth muscle cells. Furthermore, ligation of CD44 induces activation of both inflammatory and vascular cells. [10] In the present study, CD44 expression was higher in a scanty concentration of lymphocytes in epithelial-connective junction in OSMF cases, as well as it was the same event in OSCC cases.

In the present study, CD44 and ALDH1 proteins are detected in the decreased local immune response in premalignant lesion and oral malignancy. It could be suggested that these markers actively participate in plasticity and heterogeneity of microenvironment in the absence of immune response. Decreased tumor-host immune reaction revealed the prominent expression of tumor stem cell markers. Cancer chemotherapy should be used to eliminate the cancer stem cell niche.

Relationship of pathological variance and expression of markers

In OSMF, both markers tend to be seen higher positivity rate in accordance with scanty lymphocyte amount in ECJ and muscle atrophy. In OSCC, they were also associated with scanty lymphoplasmacytic infiltration around the tumor cells and Bryne's tumor classification grade III.

In the present study, CD44+ and ALDH1+ cells were detectable in Bryne's grade III tumor classification. This finding was compatible and consistent with Qian's (2013) study. [11] According to this finding, correlation between CD44 and lymphocytic infiltration was important role in inflammatory process, tumor initiation and tumor metastasis.

ALDH1 and CD44 proteins were expressed in the majority of OSCCs and majority of OSMFs. Consequently, ALDH1 and CD44 could also be expressed in the CSCs of these tumors. As a CSC marker, CD44 was more often expressed in the tumors than ALDH1. However, a lot of OSCCs expressed CD44 in almost all tumor cells and all surface epithelium of OSMFs expressed more CD44. To identify CSCs, the markers must isolate CSCs from the tumor bulk. ALDH1 was a better marker to define a subpopulation of tumor cells. Finally, the two markers were not sufficient to isolate the CSCs from the bulk of tumor cells and bulk of premalignant lesions. Further CSC markers should be used to define and isolate the CSC population.

In addition, ALDH1A1 und CD44 expres¬sion did not completely overlap. In the majority of tumors ALDH1+/CD44+, ALDH1+/CD44 and ALDH1 /CD44+ populations were observed. This obser¬vation may indicate that different CSC populations could exist within one tumor. This finding favored and followed the theory of different CSC phenotypes in HNSCC.

In conclusion, CD44 and ALDH1 appear to be important factors in carcinogenesis and tumor progression in OSMF and OSCC. It could be

suggested that loss of CD44 expression in OSMF can be considered an early event in carcinogenesis and a marker of major alterations of CD44 expression in premalignant lesions. Positivity of both markers was directly associated with Bryne's tumor grade and inversely related with tumor-host immune reaction. ALDH1 and CD44 may be expressed in the CSCs of most examined tumors and OSMF. However, these markers are not sufficient to precisely isolate the CSC subpopulation from the tumor bulk.

ALDH1 and CD44-positive cells in epithelium of OSMF and in adjacent non-tumor epithelium as well as tumor portion of OSCC suggests that changes were already underway, as these enzymes tend to be present in cells with a high tumorigenic potential. CD44 and ALDH1 appear to be important factors in carcinogenesis and tumor progression in OSCC and an important role in pathogenesis of OSMF. Positivity of both markers was directly correlated with Bryne's tumor grade and inversely correlated with tumor-host immune reaction. CD44 and ALDH1 may be expressed in the CSCs of most examined tumors and OSMF.

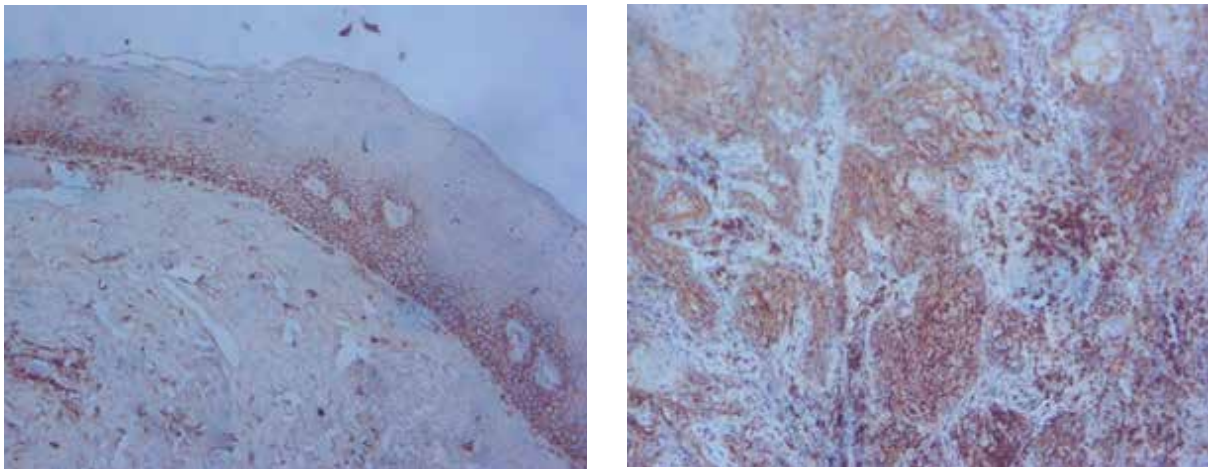

Figure 1. IHC expression of CD44 in OSMF and OSCC (400 x magnifications).

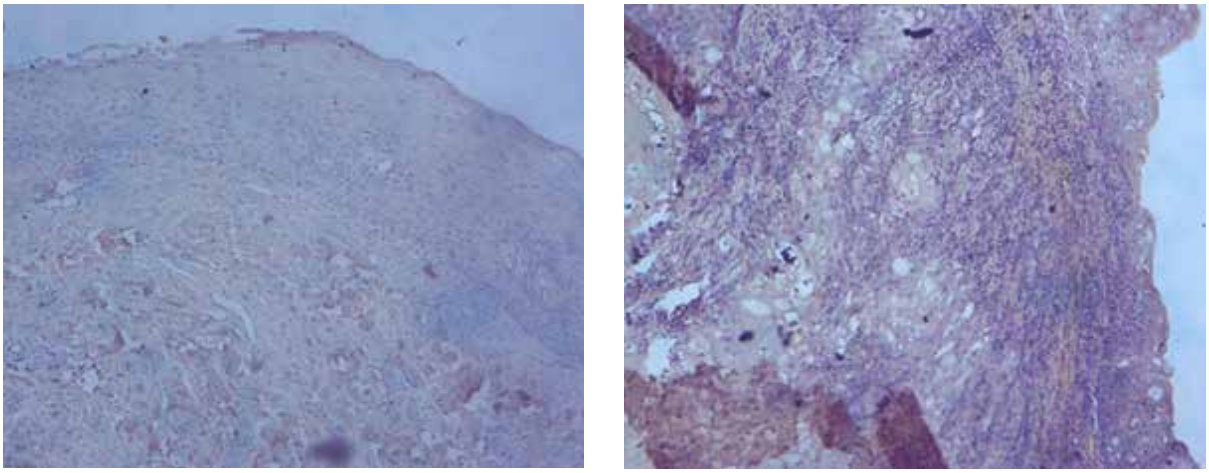

Figure 2. IHC expression of ALDH1 in OSMF and OSCC (400 x magnifications).

Table 1. CD44 and ALDH1 expression in OSMF cases (n = 30)

Expression	CD44 and/or ALDH1 expression in OSMF cases, n (%)			
	CD44+	ALDH1+	CD44+/ALDH1+	CD44-/ALDH1-
General	24(80)	17 (56.67)	17(56.67)	6(20)
Score 4CD44+	20(83.3)	14(82.35)	14(82.35)	-
Score 3CD44+	2(8.33)	2(11.76)	2(11.76)	-
Score 2CD44+	2(8.33)	1(5.88)	1(5.88)	-
Score 1CD44-	-	-	-	6(100)
Score 4ALDH1+	4(16.67)	4(23.53)	4(23.53)	-
Score 3ALDH1+	1(4.17)	1(5.88)	1(5.88)	-
Score 2ALDH1+	12(50)	12(70.59)	12(70.59)	-
Score 1ALDH1-	7(29.17)	-	-	6(100)

Table 2. CD44 and ALDH1 expression in OSCC cases (n = 34)

Expression	CD44 and/or ALDH1 expression in OSCC cases, n (%)			
	CD44+	ALDH1+	CD44+/ALDH1+	CD44-/ALDH1-
General	20 (58.82)	19 (55.88)	14(41.18)	9(26.47)
Score 4CD44+	8(40)	7(36.84)	7(50)	-
Score 3 CD44+	3(15)	2(10.53)	2(14.29)	-
Score 2CD44+	9(45)	5(26.32)	5(35.71)	-
Score 1CD44-	-	5(26.32)	-	9(100)
Score 4ALDH1+	-	-	-	-
Score 3ALDH1+	6(30)	7(36.84)	6(42.86)	-
Score 2ALDH1+	8(40)	12(63.16)	8(57.14)	-
Score 1ALDH1-	6(30)	-	-	9(100)

Table 3. Evaluation of pathological variance and expression of CD44 and ALDH1 in OSMF Cases (n = 30)

Pathologic variance	+/-	n	Evaluation of patient data and pathological variance in OSMF cases, n (%)					
			CD44+		ALDH1+		CD44+/ ALDH1+	CD44-/ ALDH1-
			(+)	(-)	(+)	(-)		
Lymphocytes in ECJ	P	12	8(66.6)	4(33.3)	6(50)	6 (50)	6 (50)	4 (33.33)
	N	18	16(88)	2(11.1)	11(61)	7(38.8)	11 (61.11)	2 (11.11)
Hyalinization	P	29	23(79)	6(20.6)	17(58)	12(41)	17 (58.62)	6 (20.69)
	N	1	1(100)	-	-	1(100)	-	-
Compressed vessel	P	23	17(73)	6(26)	12(52)	11(47)	12 (52.17)	6 (26.09)
	N	7	7(100)	-	5(71)	2(28)	5 (71.43)	-
Fibroblasts	P	8	7(87.5)	1 (12.5)	6(75)	2 (25)	6 (75)	1 (12.5)
	N	22	17(77)	5(22.7)	11(50)	11 (50)	11 (50)	5 (22.27)
Lesser vessel	P	5	5(100)	-	2(40)	3 (60)	2 (40)	1 (20)
	N	25	19 (76)	6 (24)	15(60)	10 (40)	15 (60)	5 (20)
Muscle atrophy	P	10	10(100)	-	7(70)	3 (30)	7 (70)	-
	N	20	14 (70)	6 (30)	10(50)	10 (50)	10 (50)	6 (30)

(P = Positive, N = Negative)

Table 4. Evaluation of pathological variance and expression of CD44 and ALDH1A1 in OSCC cases

Pathological variance	n	Evaluation of patient data and pathological variance in OSCC cases, n (%)					
		CD44+		ALDH1+		CD44+/ ALDH1+	CD44-/ ALDH1-
		(+)	(-)	(+)	(-)		
Keratinization							
>50%	6	5(83.33)	1(16.67)	4(66.67)	2 (33.33)	4 (66.67)	1 (16.67)
20-50%	14	7 (50)	7 (50)	8(19.51)	6 (42.86)	4 (28.57)	3 (21.43)
5-20%	13	7(53.85)	6(46.15)	6(46.15)	7 (53.85)	5 (38.46)	5 (38.46)
0-5%	1	1 (100)	-	1 (100)	-	1 (100)	-
Nuclear Polymorphism							
Little	1	1 (100)	-	-	1 (100)	-	-
Mod. abundant	19	11(57.8)	8(42.11)	13(68.4)	6 (31.58)	8 (42.11)	3 (15.79)
Abundant	13	7(53.85)	6(46.15)	5(38.46)	8 (61.54)	5 (38.46)	6 (46.15)
Extreme	1	1 (100)	-	1 (100)	-	1 (100)	-
Mitosis							
0-1	14	6(42.86)	8(57.14)	5(35.71)	9 (64.29)	1 (7.14)	6 (42.86)
2-3	17	12(70.5)	5(29.41)	11(64.7)	6 (35.29)	11(64.71)	3 (17.65)
4-5	3	2(66.67)	1(33.33)	3 (100)	-	2(66.67)	-
>5	-	-	-	-		-	-
Pattern of Invasion							
Score 1	6	5(83.33)	1(16.67)	5(83.33)	1 (16.67)	4 (66.67)	-
Score 2	2	2 (100)	-	-	2 (100)	-	-
Score 3	12	5(41.67)	7(58.33)	6 (50)	6 (50)	4 (33.33)	3 (25)
Score 4	14	8(57.14)	6(42.86)	8(57.14)	6 (42.86)	6 (42.86)	6 (42.86)
Lympho-plasmacyte*							
Marked	9	4(44.44)	5(55.56)	4(44.44)	5 (55.56)	2(22.22)	3 (33.33)
Moderate	10	5 (50)	5 (50)	3 (30)	7 (70)	2 (20)	3 (30)
Slight	14	11(78.5)	3(21.43)	11(78.5)	3 (21.43)	10(71.43)	3 (21.43)
Non-	1	-	1 (100)	1 (100)	-	-	-
Bryne's Classification							
Grade I	13	9(69.23)	4(30.77)	8(61.54)	5 (38.46)	6 (46.15)	2 (15.38)
Grade II	15	6 (40)	9 (60)	7(46.67)	8 (53.33)	4 (26.67)	6 (40)
Grade III	6	5(83.33)	1(16.67)	4(66.67)	2 (33.33)	4 (66.67)	1 (16.67)

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