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THE ROLE OF CD200 IN DIFFERENTIATION BETWEEN CHRONIC LYMPHOCYTIC LEUKEMIA AND OTHER MATURE B-CELL NEOPLASMS IN SUDANESE PATIENTS

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ABSTRACT
CD200 has been identified as an immunoregulatory molecule that is frequently elevated in various types of cancers. This study investigated the role of CD200 in differentiation between chronic lymphocytic leukemia and other mature B-Cell neoplasms. Data was collected from flow cytometer center in Khartoum state, 150 samples selected randomly. Results showed chronic lymphocytic leukemia was more incidences (63.3%) than other mature B-Cell neoplasms (36.7%). Positivity of the CD200 was not significantly affected by specimens type and gender (P.value 0.716, 0.417) for blood and bone marrow and (P.value 0.756, 0.632) for Male & Female respectively. CD200 expressed in (94.7%) of chronic lymphocytic leukemia patients comparing with (34.5%) of other mature B-Cell neoplasms. The expression of CD200 was negative in majority cases of Mantle cell Lymphoma and positive in all cases of hairy cell leukemia and these both with chronic lymphocytic leukemia were CD5 positive, so CD200 in combination with CD23 can differentiate these subtypes. There was highly significant variation of expression in CD200 between chronic lymphocytic leukemia and other mature B-Cell neoplasms (P.value 0.00). The important results were the correlation between positivity of CD200 and score system which showed significant different (P.value 0.000), when used in combination can increased the sensitivity to (94.7%). This is important in the identification of mature B-Cell neoplasms patients especially those with few numbers of immature cells seen more characteristic in mature B-Cell neoplasm than chronic lymphocytic leukemia cases. So CD200 and the scoring system were useful in differentiation between chronic lymphocytic leukemia and other mature B-Cell neoplasm.

KEYWORDS: CD200, chronic lymphocytic leukemia, mature B-Cell neoplasm.

INTRODUCTION
The World Health Organization in 2008 classification of hematologic malignancies utilizes clinical, morphologic, immunophenotypic, and genetic data to define distinct and provisional entities based on cell lineage.[1]

Lymphoid neoplasms are broadly classified into precursor lymphoid neoplasms and mature B-cell, T-cell, or natural killer (NK) cell neoplasms. CD200 is a type I transmembrane molecule and a member of the immunoglobulin supergene family.[2] This glycoprotein is not expressed by normal plasma cells and NK cells. But expressed by various cell types, including B cells, a subset of T cells (including activated T cells), thymocytes, endothelial cells, and neurons.[3] CD200 receptor 1 (CD200R1)[4], which is expressed specifically in granulocytes and monocytes and in a subset of T cells.[5-6]

Different groups have reported the strong expression of CD200 on hairy cell leukemia (HCL) cells and its potential usefulness in the differential diagnosis of CD5+ B-cell lymphomas, especially chronic lymphocytic leukemia (CLL) and mantle cell lymphoma (MCL).[7] Brunetti et al showed increased CD200 expression in HCL. Subsequently, others using flow cytometry.[8]

This study discussed the role of CD200 in differentiation between CLL and other mature B cell neoplasms (MBCNs). the immuonophenotyping markers used to

diagnose and classify were (CD5, CD23, CD22, CD79b, FMC7, Kappa and Lambda) and CD200 marker.

MATERIAL AND METHODS

Study design

This was a prospective, descriptive analytical study design based on peripheral blood and bone marrow samples from patients with mature B-cell neoplasms in flow cytometer center in Khartoum state.

Study area

This study was done in flow cytometer center in Khartoum state in which patients were coming from different states of Sudan. Khartoum, is the capital of Sudan, the Khartoum population is a fair representation of the general population of the entire Sudan.

Study population

Patients with mature B-cell neoplasms attended the outpatient flow cytometer center in Khartoum state.

Inclusion criteria

Any patients either lives in Khartoum state or come from outside for investigation in outpatient flow cytometer center and he/she have mature B-cell neoplasms included in the study.

Exclusion criteria

Any patients either lives in Khartoum state or come from outside for investigation in outpatient flow cytometer center he/she have any disease other than mature B-cell neoplasms excluded from the study.

Study duration

The study was carried out in duration between June 2015 to December 2017.

Sample size

150 samples were selected randomly from patients with mature B-cell neoplasms came to outpatient flow cytometer center.

Sample processing method

Fresh human venous blood or bone marrow mononuclear cells were isolated for investigation by aseptic international standardized techniques.

a- Venous blood sample: 2.5 ml was collected in EDTA VA container.

b- Bone marrow aspiration: 2 ml was collected in Lithium Heparin VA container.

Methodology to treat sample for flow cytometer

HISTOPAQUE-1077 technique for mononuclear cells:

1- To a 15-ml conical centrifuge tube, 3.0 ml HISTOPAQUE-1077 was added and bring to room temperature.

2- The 2.5 ml whole blood or aspirate was carefully added in the surface of the HISTOPAQUE-1077. The suspension was centrifuged at 400 x g for exactly 30 minutes at room temperature.

3- After centrifugation, with a Pasteur pipette, the upper layer was carefully aspirated to within 0.5 cm of the opaque interface containing mononuclear cells. Upper layer was discarded.

4- The opaque interface was carefully transferred with a Pasteur pipette into a clean conical centrifuge tube.

5- 10 ml of Isotonic Phosphate Buffered Saline Solution was added to this tube (step 4) and mixed by gentle aspiration.

6- All tubes were centrifuged at 250 x g for 10 minutes.

7- The supernatant was aspirated and discarded.

8- The cells pellet was re-suspended with 5.0 ml Isotonic Phosphate Buffered Saline Solution and mixed by gentle aspiration with a Pasteur pipette.

9- All tubes were centrifuged at 250 x g for 10 minutes.

10- Steps 7, 8 and 9 were repeated, the supernatant was discarded and the cells pellet was re-suspended in 0.5 ml Isotonic Phosphate Buffered Saline Solution.

Lysing procedure for bone marrow aspiration monoclonal antibody combination

1- The tubes were labeled for analysis.

2- 20 uL of monoclonal antibody was added into each tube.

3- 100 uL of sample was added containing no more than 1 x 10 leukocytes / ml. (Counted by hematology analyzer - SYSMEX).

4- Each tube was vortexes for 5 seconds.

5- Each tube was incubated at room temperature (18-25 C) for 15 minutes.

6- 3 mL of Isotonic Phosphate Buffered Saline Solution (PBS) was added to this tube (step 4) and mixed by gentle aspiration.

7- All tubes were centrifuged in 150 x g for 5 minutes and the supernatant were discarded by aspiration.

8- The pellets were Re-suspended by addition of 0.5 to 1 mL of 0.1% formaldehyde.

9- All tubes were vortexed for 5 seconds.

10- All tubes were analyzed by the flow cytometer.

Procedure for whole blood monoclonal antibody combination

1- The tubes were labeled for analysis.

2- 20 uL of monoclonal antibody was added into each tube.

3- 100 uL of sample was added containing no more than 1 x 10 leukocytes / ml. (Counted by hematology analyzer - SYSMEX).

4- Each tube was vortexed for (5 seconds).

5- Each tube was incubated at room temperature (18-25 C) for (15 minutes).

6- Add 1 ml of the "fix-and-lyse" mixture was added to the tube and vortexed immediately for three seconds.

7- Each tube was incubated at room temperature for at least 10 minutes and was protected from light.

8- Centrifuge the tube at 150 x g for 5 minutes and discard the supernatant by aspiration.

9- 3 mL of PBS was added.

10- All tubes were centrifuged in 150 x

g for 5 minutes and the supernatant were discarded by aspiration.
11- The pellets were Re-suspended by addition of 0.5 to 1 mL of 0.1% formaldehyde.
12- All tubes were vortexed for 5 seconds.
13- All tubes were analyzed by the flow cytometer.

Practice procedure
Immunostaining monoclonal antibodies were CD5/CD19, CD20, CD22, CD23, CD79b, FMC7, immunoglobulin (Kappa and Lambda) and CD200. Immunostaining monoclonal antibodies.

Conjugated mouse immunoglobulins of the IgG class were used as negative controls. Immunophenotypic analysis was performed on BECKMAN COULTER EPICS XL flow cytometer using a lymphocyte gate by forward and side scatter characteristics of the cells. A marker was considered positive when expressed more than 30% of cells above the control result. The intensity of markers were estimated and compared with the isotype control. The criteria adopted were dim, that mean the intensity was less than 1.5; and bright when the mean intensity was more than 1.5.

Result interpretation
Results were interpreted in tables and figures.

Data analysis
The data were compared by using Statistical analysis perform with Statistical Package for Social sciences (SPSS-s) version-20. In all statistical analysis, only P.value≤0.05 was considered significant. Following statistical measures were used. Mean, Standard SD, and percentage. Chi-test and correlation (Pearson Correlation) were used (significance level were set at P≤ 0.05).

Ethical consideration
The patient inclusion in the study was voluntary, after taken his written consent. Informed consent was attached to each questionnaire to be obtained from the patient. There was full commitment standard precaution for sample taken, privacy and confidentiality.

RESULTS AND DISCUSSION
The statistical results of the study showed that males were affected more than females (72.7%, 27.3%) respectively; (Figure 1), this finding agreed with. Altayeb O and Ansell P et al studies whose reported that most NHLs are common in men[9-10], Gujral S et al also reported that the men affected more than women with a ratio M:F (4:1).[11]

The average mean age of patients with mature B-cell neoplasms in the study was (65 years), which means that the mature B-cell neoplasms are generally occurred in the age groups of relatively older ones, to some extent, this finding consist with Altayeb O and Ansell P et al results whose reported that median age at diagnosis of most patients exceeds (60.7 and 70 years) respectively.[9-10]

Also the result was similar to Sandes AF which found that the median age was (65 years).[7]

Patient's samples included both bone marrow aspirate (BM) and peripheral venous blood (PB), where the frequency of each type were (22.7% and 77.3%) respectively (Figure 2).

Concerning the diagnosis, the majority of patients were CLL, which were (63.3%). They represented the higher frequency than the cases of other MBCNs (36.7%) (Table 3). This finding consisted with Altayeb O and Gujral S et al studies, who's reported that when using flow cytometry immunophenotyping; CLL was the commonest subtype (66.5% and 68.5% respectively).[9,11]

CD200
When examining the frequency of CD200 remark in CLL, the positivity was (94.7%), while its negativity was (5.3%) (Table 1) compared to other MBCNs which was (34.5%) positive remark and ((34.5%) negative for CD200 remark (Table 2). The high expression percentage result of CD200 remark in CLL was similar to study done by Rahman K who stated that all cases of CLL (100%) were positive for CD200 remark.[12]

This study showed there was no significant variation between male and female in mean positivity (remark) and mean florescent intensity of CD200 with P.value (0.716 and 0.756) respectively (Table 5, 6).

The samples type in the study either peripheral blood or bone marrow aspirate, and both showed no significant variation in mean positivity (remark) of CD200% and mean florescent intensity of CD200 with P.value (0.417 and 0.632), respectively. (Table 7, 8). So peripheral blood sample was better than bone marrow aspirate for investigation of CD200, because the first was simple and easy but the latter was invasive technique.

For how CD200 differentiate between CLL and other mature B. cell neoplasms which was main objective of the study; There was highly significant variation on expression of CD200 between CLL and other NHL (P.value 0.000) (Table 3). On the other hand there was no significant variation in mean florescent intensity of CD200 according to diagnosis P.value (0.106) (Table 4).

CD200 Expression in CLL
As we mentioned above nearly all cases of CLL (94.7%) were positive for CD200, the remaining (5.3%) were negative In nearly (85.3%) of cases were bright CD200 expression (i.e., had a CD200 Mean Fluorescent Intensity (MFI) of more than 1,500), and (14.7%) of cases were negative or dim CD200 expression (i.e., had a CD200 MFI of less than 1,500). The expression of mean fluorescent intensity of the study was similar to other

studies reported by Challagundla P and Sandes AF showed that the expression of CD200 on CLL was ranged from bright to highly expressed.[7,13]

This study showed CD200 Expression in MCL (85.7%) were negative indicated by dim CD200 expression, the remaining (14.3%) were positive with bright expression. Consistent with study done by Challagundla P which reported mantle cell lymphoma (MCL) cases were usually dim or negative.[13] However, rare MCL cases (about 5%) were moderately bright for CD200, and also similar to Sandes AF study which stated that all MCL cases were negative for CD200 expression.[7]

CD200 Expression in HCL

The study showed all cases of hairy cell leukemia were positive for CD200 with bright CD200 expression. The present study similar to Sandes AF in which reported that all cases of HCL was positive for CD200 remark and brightly expressed.[7]

CD200 Expression in Diffuse Large B-Cell Lymphoma (DLBCL)

Cases of DLBCL have heterogeneous characteristics for CD200 expression, (73.1%) of cases were negative for CD200 with dim expression and (26.9%) were positive, majority (62.5%) with dim expression.

CD200 Expression in Other B-Cell Neoplasms

Among the other B-cell lymphomas and leukemia, positive CD200 with bright CD200 expression was observed in (71.4%) of splenic marginal zone lymphoma (MZL) cases, but negative CD200 with dim expression in all cases of prolymphocytic leukemia (PLL) and follicular lymphoma (FL). The study PLL results similar to Sandes AF which reported (71.4%) of PLL was negative to CD200. Scoring system and pan B-cell markers:

CD20 and CD19

CD20 and CD19 expressed in all types of mature B-cell neoplasms, both have role in the differentiation between mature B-cell neoplasms and other blood cell neoplasms. In the study nearly all samples were positive (98.7% and 99.4%) for CD19 and CD20 respectively.

CD5

It considered one of the most important markers help in the diagnosis and classification of mature B-cell neoplasms. When examining the CD5 in mature B-cell neoplasms cells both in CLL and NHL, as general, we found that there were (81.1%) positive and (18.9%) negative for CLL with mean remark (67.5%), while there were (25.5%) positive and (74.5%) negative for other NHL with mean remark (32.9%), this positivity percentage of NHL mainly in the MCL cases (all cases in this study) this gives more support in the diagnosis of MCL.

CD23

The marker often appear in CLL and rarely in cases of NHL; as the CD23% expressed in the (85.3%) of CLL cases with mean remark (57.7%), but with other NHL only expressed in two cases (3.6%) and didn't in (96.4%) with mean remark (5.4%). It’s one of the scoring system and preferred always to couple with CD5 especially it doesn't express by T cells, it's also important to know the CD23 not express in cases of MCL so the using of both CD23 and CD5 increase and facilitate to differentiate between CLL and MCL which both positive CD5 especially when the points of scoring markers are falling between CLL and NHL points, In this case, the demand to using the specific markers to distinguish between CLL and MCL (The solution will be in CD200).

CD22

It is one of the important markers in the diagnosis of MBCN. The importance comes from the significant role in the differentiation between CLL and NHL. It was negative in (77.9%) of CLL cases, and positive in (22.1%), with mean remark (18.8%), while in NHL cases it was positive in (81.8%) and negative in (18.2%) with mean remark (63%) (Table 12,13).Therefore, it was unlike CD5 and CD23 which appear more in NHL cases, CD22 showed more positivity in NHL cases, this study consistent with Altayeb O which reported CD22 negative in (93%) of CLL cases, and positive in (96%) of NHL cases.[9]

CD79b

It was positive in (32.6%) and negative in (67.4%) for CLL with mean remark (26.1%), but for NHL it was positive in (94.5%) and negative in (5.5%) with mean remark (74%) (Table 12,13). The findings were similar to Altayeb O results which showed that CLL (29.0%) were positive for CD79b while it was (98.2%) in NHL.[9]

FMC7

It was negative in (91.6%) of CLL cases, and positive in (8.4%), with mean remark (13.2%), while in NHL cases it was positive in (89.1%) and negative in (10.9%) with mean remark (67%) (Table 12, 13). The study was similar to Altayeb O which showed FMC7 were negative in (89.1%) of CLL cases, and positive in (99.5%) of NHL cases.[9] So the FMC7% represent one of the most important markers for differentiation between CLL and NHL, this importance resulting from appeared with high percentage in cases of NHL while disappeared in majority CLL cases, these results were compatible with Matutes et al study who confirmed that the absence of expression of FMC7 was one of the most reliable markers that differentiated CLL from other B-cell neoplasm.[14]

Immunoglobulins (Kappa & Lambda)

Kappa was negative in (92.6%) of CLL cases, and positive in (7.4%), with mean remark (8.2%), while in NHL cases it was positive in (52.7%) and negative in (47.3%) with mean remark (42.3%) (Table 12, 13), this

result in comparison with Altayeb O reported near to his findings which was (18.6%) positive and (81.4%) negative in CLL.[9]

Lambda was negative in (95.8%) of CLL cases, and positive in (4.2%), with mean remark (5.3%), while in NHL cases it was positive in (20%) and negative in (80%) with mean remark (14%) (Table 12, 13). This study showed that cases of CLL which expressed Ig which expressed Kappa restriction were same as Lambda restriction, also the percentage of Ig in CLL cases always low, so we can say these are the characteristics of the Ig in the cases of MBCN, the Ig appeared in NHL cases always were high percentage.

Therefore, Kappa and Lambda showed role in the differentiation between CLL and NHL.

Sensitivity and specificity

Sensitivity is very important in the identification of MBCN patients especially in cases which have few numbers of immature cells seen more characteristic in NHL than CLL.

After studying of scoring markers and CD200, arranged gradually from the highest; Lambda gave the highest sensitivity (95.8%), CD200 (94.7%), Kappa (92.6%), FMC7 (91.6%), CD23 (85.3%), CD5 (80%), CD22 (77.9%) and the lowest was CD79b (66.3%) (Table 15).

Concerning the specificity, it has a vital role to know the ability of markers to differentiate between CLL and NHL. This specificity means the ability of the marker to predict certain type of NHL. After studying the results of specificity for the scoring parameters and CD200, CD23 was the highest specificity (96.4%), then CD79b (94.5%), FMC7 (89.1%), CD22 (81.8%), CD5 (74.5%), CD200 (65.5%), Kappa (52.7%) and the lowest was Lambda (20%) (Table 15).

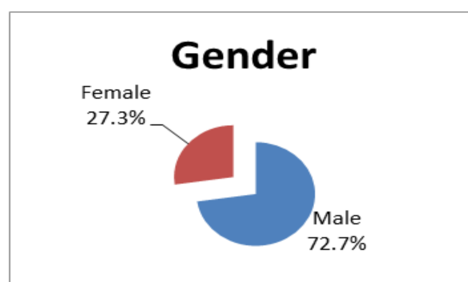

Figure (1): Frequency of mature B-cell neoplasm according to gender.

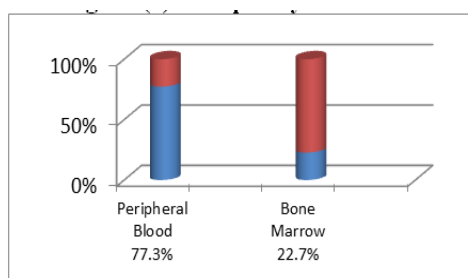

Figure (2): Shows frequency of peripheral blood and bone marrow aspiration specimens in the study.

Table (1): Frequency of CD200 positivity among CLL cases.

CLL	Frequency	Percent	Range
Positive	90	94.7	31.1 – 97.5
Negative	5	5.3	
Total	95	100.0	

Table (2): Frequency of CD200 positivity among NHL cases.

NHL	Frequency	Percent	Range
Positive	19	34.5	36.7 – 94.1
Negative	36	65.5	
Total	55	100.0	

Table (3): Shows positivity of CD200 in relation to mature B-cell neoplasms.

Disease	N	Mean	SD
CLL	95	78.0	22.7
NHL	55	30.3	34.3

Table (4): Shows florescent intensity of CD200 among mature B-cell neoplasms.

Disease	N	Mean	SD
CLL	95	2.7	1.3
NHL	55	2.1	2.7

Table (5): Shows positivity of CD200 according to gender.

Gender	N	Mean	SD
Male	109	59.9	35.6
Female	41	62.3	36.9

Table (6): Shows florescent intensity of CD200 according to gender.

Gender	N	Mean	SD
Male	109	2.5	2.1
Female	41	2.4	1.2

Table (7): Mean positivity of CD200 according to specimen type (Peripheral Blood or Bone Marrow aspirate).

Specimen	N	Mean	SD
PB	116	59.2	36.8
BM	34	64.9	32.4

Table (8): Shows florescent intensity of CD200 according to specimen type (Peripheral Blood or Bone Marrow aspirate).

Specimen	N	Mean	SD
PB	116	2.4	2.0
BM	34	2.6	1.4

Table (11): CD200 expression (positivity and mean fluorescent intensity) among mature B-cell neoplasms.

MBCN	CD200 Remark		CD200 MFI	
	Positive %	Negative %	Bright	Dim
CLL	94.7	5.3	85.3	14.7
MCL	14.3	85.7	42.9	57.1
HCL	100	0	100	0
DLBCL	30.8	69.2	37.5	62.5
FL	0	100	0	100
SMZL	71.4	28.6	100	0
PLL	0	100	0	100

Table (12): Frequency of scoring markers positivity; CD5, CD23, CD22, CD79b, Kappa, Lambda and FMC7 among CLL cases.

Scoring marker	CLL	
	Positive Percent	Negative Percent
CD5	81.1	18.9
CD23	85.3	14.7
CD22	22.1	77.9
CD79b	32.6	67.4
Kappa	7.4	92.6
Lambda	4.2	95.8
FMC7	8.4	91.6

Table (13): Frequency of scoring markers positivity; CD5, CD23, CD22, CD79b, Kappa, Lambda and FMC7 among NHL cases.

Scoring marker	NHL		Total
	Positive Percent	Negative Percent	
CD5	25.5	74.5	100
CD23	3.6	96.4	100
CD22	81.8	18.2	100
CD79b	94.5	5.5	100
Kappa	52.7	47.3	100
Lambda	20	80	100
FMC7	89.1	10.9	100

Table (14): Frequency of CD200 positivity regarding score.

Score	CD200 Remark (Positivity)		Total
	Positive Percent	Negative Percent	
0.0	41.7	58.3	100
0.5	50	50	100
1.0	30	70	100
1.5	28.6	71.4	100
2.0	50	50	100
2.5	0	100	100
3.0	100	0	100
3.5	83.3	16.7	100
4.0	86.5	13.5	100
4.5	100	0	100
5.0	100	0	100

Table (15): Markers Specificity and sensitivity.

Marker	Specificity	Sensitivity
CD200	65.5	94.7
CD23	96.4	85.3
CD5	74.5	80
CD22	81.8	77.9
CD79	94.5	66.3
Kappa	52.7	92.6
Lambda	20	95.8
FMC7	89.1	91.6

CONCLUSION

CD200 is expressed in mature B-cell neoplasms. Analysis of its expression has several diagnostic applications in the flow cytometric evaluation of these lymphoid malignancies. However, a significant immunophenotypic overlapping occurs between subtypes of mature B-cell neoplasms especially between CLL, MCL and HCL cells which all classified as CD5+. In this study, we investigated the expression of recently identified marker CD200 in mature B-Cell neoplasms patients. We have confirmed previous reports that CD200 is consistently expressed in nearly all CLL and all HCL, but not expressed in MCL, so if needed to diagnosis CD5 NHL it's better to couple with CD23, and finally CD200 positivity strongly suggests the diagnosis of CLL. The inclusion of CD200 in the MBN routine flow cytometry panels facilitates the differentiation of CLL, HCL and MCL and has a great role in diagnosis accuracy and precision of these subtypes of mature B-cell neoplasms.

RECOMMENDATION

This was the first study that demonstrated the applicability of CD200 in mature B-cell neoplasms diagnosis in Sudan.

The study recommended to introduced CD200 as routine marker in Sudan for differentiation between CLL and other mature B-cell neoplasms, because it is more reliable when used with the scoring system markers in certain cases for precise diagnosis. So it increased the sensitivity and specificity.

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