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ANALYSIS OF DIFFUSE LARGE B-CELL LYMPHOMA (DLBCL) MICROENVIRONMENT IN REGARD TO DRUG RESISTANCE PATTERNS

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Abstract:

Lymphomas are one of the most common hematological malignancies, characterized into Hodgkin's Lymphoma (HL) and Non-Hodgkin's Lymphoma (NHL). Diffuse Large B-cell Lymphoma (DLBCL) is the most common type of NHL, often seen in patients diagnosed with genetic immunological deficiencies. It accounts for nearly one third of all the new cases of NHL. Tumor Microenvironment (TME) has helped to explain the heterogeneous nature of DLBCL and has a surfeit of immune cells, blood vessels, extracellular matrix (ECM) and stroma cells, influencing the tumor behavior and subsequently drug resistance. This study aimed to analyze selective drug resistant marker panel; MDR1 and EMT (E- Cadherin, Vimentin and SNAI1) to predict their role in tumor microenvironment of DLBCL. Using blood samples of 28 patients, total RNA was extracted by TRIzol method and cDNA synthesis was performed. DNA primers were designed using bioinformatics tools and optimized using conventional PCR. Further, quantification of drug resistant markers was carried out using RT-qPCR. The results showed an insignificant expression of MDR 1 in DLBCL patients suggesting sensitivity to the drug being administered. In DLBCL, the expression of E-Cadherin was higher than Vimentin and SNAI-1 suggesting there was no progression towards epithelial to mesenchymal transition (EMT).On the contrary, nodal marginal zone lymphoma (NMZL) showed a significantly higher expression of Vimentin and SNAI-1 indicating progression of EMT. The expression of these biomarkers in association to drug resistance, can help us gain a better understanding of the tumor microenvironment. Contributing to improved prognosis and therapeutic methods in aggressive tumors.

Keywords: Diffuse Large B-Cell Lymphoma (DLBCL), Hematological Malignancy, Drug Resistance, Tumor Microenvironment, Chemotherapy.

INTRODUCTION:

Lymphoma is a white cell disorder with tumors common in lymph nodes. [1] Neoplasms of lymphoid origin often aid in disrupting the normal function of immune system. Lymphomas are divided into Hodgkin's Lymphoma (HL) and Non-Hodgkin's Lymphomas (NHL). HL comprises 10% while NHL comprises 90% of the lymphomas. NHL is further divided into B-cell and T-cell subtypes. [2] Non-Hodgkin's Lymphoma (NHL) is the 4th most common cancer with male predominance in Pakistan. The NHL diagnosed in teenagers is less clinically aggressive than the one diagnosed in adults. It is broadly classified into B and T cell malignancies. [3]

Diffuse Large B-Cell Lymphoma (DLBCL):

Diffuse Large B-Cell Lymphoma (DLBCL) is the most common type of NHL and is heterogeneous in nature. According to the cell of origin (COO), it is divided into two sub- categories activated B-cell like (ABC) and germinal center B-cell like (GCB).[4] DLBCL is seen to be frequently present in patients with genetic immunological deficiencies. Along with that, an association between DLBCL and viruses has also been observed. [5]

Another important clinical entity within NHL is Richter's transformation or Richter's Syndrome (RS), which is the histological transformation of a patient with Chronic Lymphocytic Leukemia (CLL) to an aggressive lymphoma, in most cases Diffuse Large B-cellLymphoma (DLBCL). Ritcher's Syndrome occurs in approximately 15% of CLL patients who will experience a transformation to DLBCL. [6] The risk of this transformation depends on 3 factors i.e. externalfactors including viral factors or genotoxic therapy, immunological features of the host and individual tumor biology i.e. disease burden, genomic instability. [7] A chemotherapy treatment of purine analogues in combination with alkylating agents increase the risk of developing RS to three folds. [8]

Tumor microenvironment (TME) plays an important role in reshaping of tumor behavior by metabolic reprogramming, immune modulation and most important of all inducing invasion and metastasis through Epithelial to Mesenchymal Transition (EMT) and subsequently drug resistance. [9] Multidrug resistance because of EMT can be caused by ATP binding cassette (ABC) transporters. ABC transporters have been reported to be clinically relevant to MDR. MDR1 glycoprotein (ABCB1) has been identified as in multipledrug resistant cell lines. [10] EMT is series of events in which the epithelial cells lose their characteristics and obtain the properties of mesenchymal cells. [11] EMT plays an important role in cancer metastasis and is vital process in which the cells detach from their neighboring environment and invade the extracellular matrix (ECM), finally entering the circulation. [12]

E-Cadherin is a structural protein and has been known to mediate cell to cell adhesion.It is a prototype of classical cadherins. [13] E-Cadherin is one of the tumor suppressor proteins, during metastasis a loss of E-Cadherin expression in association EMT is observed. [14] E-Cadherin has been seen to inhibit receptor tyrosine kinases (RTK), which explainsthe activation of RTK's in cancer as a result of decrease in E-Cadherin expression. An overexpression of EGFR is an early event in cancer progression. While the downregulation might occur in the later stages, leading to metastasis and invasiveness in cancers. [13] Vimentin plays a significant role in tumor progression and is a mesenchymal marker. In cancers, Vimentin is generally overexpressed. It might not necessarily increase proliferation but it increases invasiveness and migration significantly. [12] SNAI-1 is a zinc finger transcription factorwhich induces EMT in normal or cancer cells. It has a highly conserved C-terminal domain i.e.4-6 C2-H2-type zinc fingers that bind to the E-Box. [15] The EMT induced by SNAI-1 induces drug resistance, invasion and immunosuppression. [16]

Multidrug resistance 1 gene encodes P-glycoprotein (P-gp), involved in the efflux of several compounds. [17] ABC transporters are one of the largest membrane transporters, and export exogenous and endogenous substrates from the cells. MDR1 is overexpressed in cancer cells. [18]). High Pgp levels in the brain can disrupt the intake of adequate amount of desired drugs and low Pgp levels might lead to excessive amount of drugs in the brain. The expression levels of MDR1 control the synthesis of the protein and the protein then determines transport of substrates from the cell. [19]

AIMS AND OBJECTIVES:

1. **Analyzing the Expression of EMT Markers (E-Cadherin, Vimentin and SNAI-1) in DLBCL Patients:**
   The aim was to observe the expression of Epithelial to Mesenchymal Transition using EMT biomarkers and their effect on tumor behavior. Focusing on drug resistance and its association with the chemotherapy regime being administered to the patients.
2. **Analyzing the Expression of MDR1 in DLBCL Patients:**
   This objective focused on the expression of Multidrug resistance 1 gene, which is an ATP binding cassette (ABC) transporter. Its overexpression is directly related to the severity of cancer. Poor uptake of drugs being administered is seen with high levels of MDR1 in cancer patients.
3. **To Observe the Role of Tumor Microenvironment on Tumor Behavior:**
   The study intended to determine the role of tumor microenvironment on the tumor behavior and drug resistance using biomarkers and a comparative analysis of DLBCL with aggressive tumor types.

LITERATURE SUPPORT:

The correlationof tumor-infiltrating macrophages (TAMs), a major component of tumor microenvironment, with prognosis of Diffuse Large B-Cell Lymphoma was studied by Cai and coworkers in 2012. They concluded that the patients who had a high expression of TAMs in their microenvironment had more invasive and metastatic cancers with a lower complete response (CR) rate and poor prognosis. [20] The role of tumor associated macrophages (TAMs) in association with epithelial to mesenchymal (EMT) phenomenon was studied by Zhang and coworkers in 2016. TAMs infiltration wasassociated with proteins related to EMT in gastric cancer. The levels of TAM infiltration can serve as a therapeutic target in cancer. [21] In support of previous studies of E-Cadherin in cancer 2013, Jordaan and his coworkersworked on assessing the re-expression of E-Cadherin in Chronic Lymphocytic Leukemia (CLL) patients. They reported that E-Cadherin is hypoacetylated and modified epigenetically in CLL cells. [22] Drug resistance in DLBCL patients is a majorcause of treatment failure. Maxwell et al., concluded from their results that Akt signaling pathway activation promotes drug resistant phenotype with Vimentin dependent invasion in DLBCL cells. [23] In the process of EMT, loss of cell to cell adhesion by the inactivation of E-Cadherin occurs. The inactivation of E-cadherin is the role of Snail/SNAI-1. Julien et al., performed immunostaining, microarray and semiquantitative real-time RT-PCRfor the gene expression on cell cultures and concluded that NF-κB induces the expression of Snail and that the NF-κB pathway activation associates with cancer progression and metastasisin several human cancers. While E-Cadherin acts as a tumor suppressing gene. [24] The expression of MDR1 gene in B-cell lymphomas was observed in a study conducted byYagi et al. The results of the study suggested that follicular dendritic cells (FDCs) induce MDR1 expression in neoplastic and reactive B-cells. [25]

METHODOLOGY:

Sample collection:

64 patients were enrolled from the oncology department of Jinnah Hospital, Lahore. An informed consent was taken from the enrolled patients. 16 patients were lost to follow up, the remaining 48 patients were divided into 7 groups along with a group of healthy individuals. 5ml of blood of enrolled subjects was collected in an EDTA vial during chemotherapy.

RNA Extraction:

RNA extraction was performed using the TRIzol method briefly. The quantity and quality of RNA was checked by using Nanodrop 2000/2000c Spectrophotometer (Thermoscientific, USA). The samples 260/230 and 260/280 ratio of more than 1.5 were selected for further cDNA processing. Others were either re-precipitated or extracted again.

cDNA Synthesis:

The synthesis of single stranded cDNA was performed by (M-MuLV) Reverse Transcriptase Kit (Catalogue # K1622, Thermoscientific, USA). The steps were followed according to the manufacturer's instruction briefly. The tubes containing the cDNA were stored at -20°C for further processing.

Primer Designing and Optimization:

Primers were designed using the NCBI primer designing tool. A set of primers was designed for each biomarker (MDR1, E-Cadherin, SNAI-1 and Vimentin). Primer optimization was done using gradient PCR thermocycler (Bio-Rad T100-Thermocycler, USA) to determine thebest Tm. For the confirmation of primers, In-Silico PCR on UCSC Genome Browser was used.

Agarose Gel Electrophoresis:

The gel was visualized under the UV trans-illuminator (GelDoc-It imaging system UVP with gel camera 300) and images were acquired.

Real Time Quantitative Polymerase Chain Reaction (RT-qPCR):

CFX 96 qPCR system by Bio-Rad was used to perform RT-qPCR to observe the expression analysis of mRNA MDR1, E-Cadherin, SNAI-1 and Vimentin. Before each primer, the RT-qPCR was optimized at annealing stage according to the Tm of respective primers.

RT-qPCR Data Analysis:

For the analysis of data obtained from the RT-qPCR Livak's method (2001) was used by analyzing relative fold change expression of the inflammatory markers. The relative expression of markers was calculated with reference to the control samples by applying the following formula:

$$\Delta Ct \text{ (sample)} = (Ct \text{ target} - Ct \text{ reference})$$
$$\Delta Ct \text{ (Calibrator)} = (Ct \text{ target} - Ct \text{ reference})$$
$$\Delta\Delta Ct = \Delta Ct \text{ calibrator} - \Delta Ct \text{ experimental}$$
$$\text{Fold increase} = 2^{-(\Delta\Delta Ct)}$$

Statistical Analysis:

For statistical analysis Graph Pad Prism Software (version 8) was used. Significant relation between two variables were assessed by unpaired t-test with 95% confidence interval. Results were expressed as mean ± SD. All the experiments were repeated multiple times and consistent reproducibility was seen. A p-value of ≤ 0.05 was considered statistically significant.

RESULTS:

In this study 48 patients of lymphomas were enrolled along with 6 healthy individuals taken as control. Subjects were divided in groups for comparative analysis between different cancers. Table 1 shows that there were 19% females and 81% males. The median age for males and females is ±18.96 and ±13.75, respectively.

Table 1: Demographic features of study population

Parameters	Groups	Gender		Mean		Range		Standard Deviation (SD)	
		M	F	M	F	M	F	M	F
Age (Years)	15-40	18	6						
	40 above	21	3	45	33	57	30	±18.96	±13.75

Table 2 shows the clinical parameters of the patients that were observed and the patients with Diffuse Large B-Cell Lymphoma were receiving RCHOP and CVI, the Hb in the patients ranged from 8.1-12, WBCs ranged from 4.4-9.3 and the platelets ranged from 320-420. The patients with Nodal Marginal Zone Lymphoma were given CVP, their blast percentage was <5%. The Hb ranged from 11.5-12, the WBCs ranged from 3.3-4 and the platelets ranged from 200-215. The patients with Non Hodgkin Lymphoma were given CHOEP, LRM and Cisplatin. Their Hb ranged from 9.4-13.7, the WBCs ranged from 2.4-11.6 and the platelets ranged from 184-702. The patients with Hodgkin Lymphoma were given Gem OX and had an Hb range of 9-11, the WBCs ranged from 4-5.2 and the platelets had an average of 316.

Table 2: Clinical parameters

Parameter	DLBCL	AL	CL	BCLL	NMZL	NHL	HL
Chemo Regimen	RCHOP,CVI	HiDAC HRM, WI	CVT,CVP, RCHOP	HCVAD	CVP	CHOEP, LRM, Cisplatin	Gem OX
Blast %	N/A	4-70%	<5%	<5%	<5%	N/A	N/A
Hb Range	8.1-12	8.7-14	9.4-12.3	9.9-11.2	11.5-12	9.4-13.7	9-11
WBC Range	4.4-9.3	5-9.7	36-132	3-4.6	3.3-4	2.4-11.6	4-5.2
Platelet Range	320-420	139-471	59-496	38-55	200-215	184-702	316

Expression of selected biomarkers through RTq-PCR was done. DLBCL showed insignificant difference of E-Cadherin expression as compared to Vimentin, which showed their non-aggressive nature. NHL and HL shows indifferent expression of EMT markers i.e. E-Cadherin and Vimentin. DLBCL showed insignificant difference of E-Cadherin expression as compared to SNAI-1. Which showed their non-confrontational nature. Other NHL and HL showed indifferent expression of EMT markers i.e. E-Cadherin and SNAI-1. Expression of MDR1 was significantly decreased in all study groups.

Significantly Increased Expression of MDR1 in BCLL Relates to Tumor Aggressiveness:

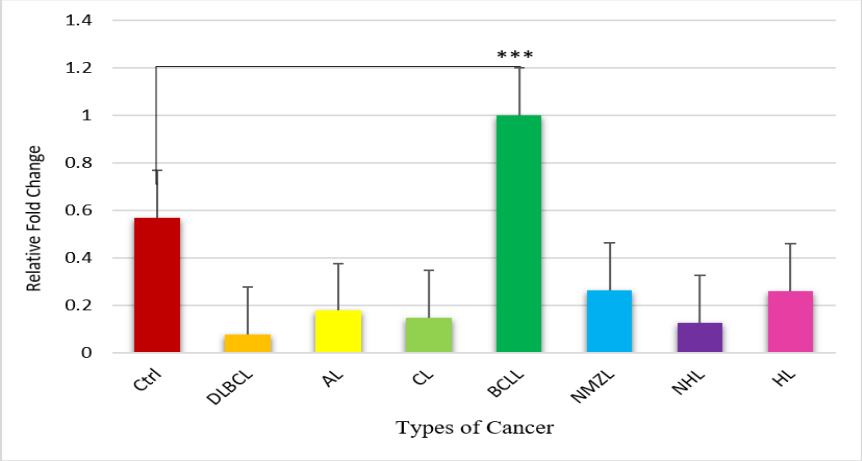

Figure 1: Gene expression profiling of Lymphoma and Leukemia patients. Control (Ctrl), Diffuse Large B-Cell Lymphoma (DLBCL), Acute Leukemia' (AL), Chronic Leukemia's (CL), B-Cell Lymphocytic Leukemia (BCLL), Nodal Marginal Zone Lymphoma (NMZL), Non-Hodgkin Lymphoma (NHL), Hodgkin Lymphoma (HL). (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$)

DLBCL, Acute and Chronic Leukemia's did not induce expression of EMT Related Drug Resistance:

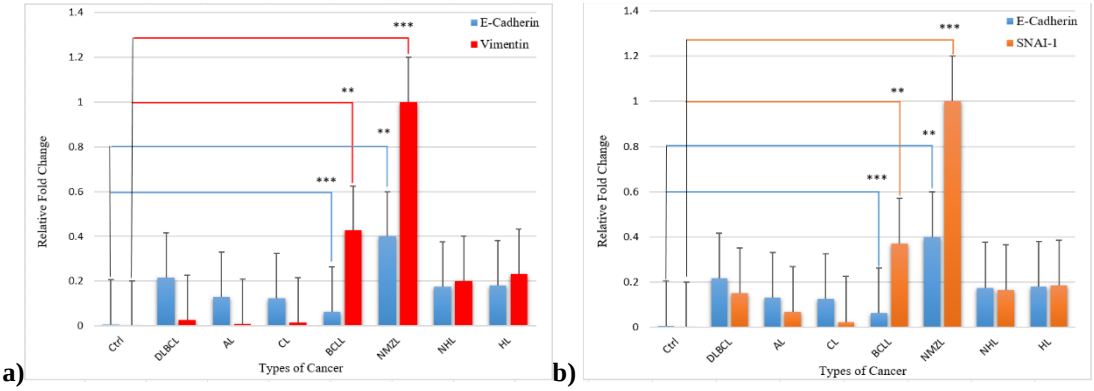

Figure 2: a) Comparative gene expression of E-Cadherin with Vimentin. Acute Leukemia's (AL), Chronic Leukemia's (CL), B-Cell Lymphocytic Leukemia (BCLL), Nodal Marginal Zone Lymphoma (NMZL), Non-Hodgkin Lymphoma (NHL), Hodgkin Lymphoma (HL). (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001)

b) Comparative gene expression of E-Cadherin with SNAI-1. Acute Leukemia' (AL), Chronic Leukemia's (CL), B-Cell Lymphocytic Leukemia (BCLL), Nodal Marginal Zone Lymphoma (NMZL), Non-Hodgkin Lymphoma (NHL), Hodgkin Lymphoma (HL). (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001)

DISCUSSION:

According to the results of this cross sectional study examining the role of tumor microenvironment in Lymphomas specifically DLBCL, drug resistant markers have shown significant role in the shaping of tumor microenvironment controlling the behavior of the cancer.

A stable expression of MDR1 in most of the cancer types was noticed in the results,showing chemotherapeutic sensitivity. Whereas high expression of MDR1 in NHL aggressive type correlates with drug resistance. As observed in the results, Diffuse Large B-Cell Lymphoma showed some level of MDR1 expression which was not significantly high. DLBCL although has low prognosis but has a lower expression of MDR1 as observed compared to other B-cell lymphomas. [25]

An inverse relation of E-Cadherin expression to Vimentin and SNAI-1 expression was observed by our results which supports the EMT transition and explains the tumor growthand metastasis in cancers. DLBCL showed an increased expression of E-Cadherin and a decrease in Vimentin expression. Lemma Sand and coworkers conducted a study of biological and prognostic features of EMT factors in DLBCL and stated that Factors like Twist and ZEBI downregulate the expression of E- Cadherin, which is an essential molecule in cell to cell adhesion. E-Cadherin has often been called a suppressor gene [26]. In another study by Maxwell and coworkers it was observed that expression of Vimentin was higher in patients who had CHOP-resistant DLBCL cells. [23] An inverse relation of SNAI-1/Snail with E-Cadherin was also observed and the results revealed significantly increased expression of EMT markers in aggressive tumors. Our study showed an inverse expression levels of E-cadherin and Snail, revealing that DLBCL was not drug resistant and was less invasive as EMT was not progressive. A study conducted by Zheng and colleagues on regulation of EMT in metastasis stated that Snail expression can be inhibited to reduce metastasis and invasion in DLBCL. [27]

REFERENCES

1) Sung H, Ferlay J, Siegel RL et al., Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin. 2021 May;71(3):209-249.
2) Mugnaini EN, Ghosh N. Lymphoma. Prim Care. 2016 Dec;43(4):661-675.
3) Thomas AG, Vaidhyanath R, Kirke R et al., Extranodal lymphoma from head to toe: part 1, the head and spine. AJR Am J Roentgenol. 2011 Aug;197(2):350-6.
4) Xu-Monette ZY, Zhang H, Zhu Fet al., A refined cell-of-origin classifier with targeted NGS and artificial intelligence shows robust predictive value in DLBCL. Blood Adv. 2020 Jul 28;4(14):3391-3404.
5) Martelli M, Ferreri AJ, Agostinelli C et al., Diffuse large B-cell lymphoma. Crit Rev Oncol Hematol. 2013 Aug;87(2):146-71.
6) Chigrinova E, Rinaldi A, Kwee I et al., Two main genetic pathways lead to the transformation of chronic lymphocytic leukemia to Richter syndrome. Blood. 2013 Oct 10;122(15):2673-82.
7) Jamroziak K, Tadmor T, Robak T et al., Richter syndrome in chronic lymphocytic leukemia: updates on biology, clinical features and therapy. Leuk Lymphoma. 2015 Jul;56(7):1949-58.
8) Parikh SA, Shanafelt TD. Risk factors for Richter syndrome in chronic lymphocytic leukemia. Curr Hematol Malig Rep. 2014 Sep;9(3):294-9
9) Erin N, Grahovac J, Brozovic A et al., Tumor microenvironment and epithelial mesenchymal transition as targets to overcome tumor multidrug resistance. Drug Resist Updat. 2020 Dec;53:100715
10) Andreadis C, Gimotty PA, Wahl P et al., Members of the glutathione and ABC-transporter families are associated with clinical outcome in patients with diffuse large B-cell lymphoma. Blood. 2007 Apr 15;109(8):3409-16.
11) Goldman O, Valdes VJ, Ezhkova E et al., The mesenchymal transcription factor SNAI-1 instructs human liver specification. Stem Cell Res. 2016 Jul;17(1):62-8.
12) Saentaweesuk W, Araki N, Vaeteewoottacharn K et al., Activation of Vimentin Is Critical to Promote a Metastatic Potential of Cholangiocarcinoma Cells. Oncol Res. 2018 May 7;26(4):605-616.
13) Andl CD, Rustgi AK. No one-way street: cross-talk between e-cadherin and receptor tyrosine kinase (RTK) signaling: a mechanism to regulate RTK activity. Cancer Biol Ther. 2005 Jan;4(1):28-31.
14) Na TY, Schecterson L, Mendonsa AM et al., The functional activity of E-cadherin controls tumor cell metastasis at multiple steps. Proc Natl Acad Sci U S A. 2020 Mar 17;117(11):5931-5937.
15) Qi J, Li T, Bian H et al., SNAI1 promotes the development of HCC through the enhancement of proliferation and inhibition of apoptosis. FEBS Open Bio. 2016 Mar 10;6(4):326-37.
16) Furuya S, Endo K, Takahashi A et al., Snail suppresses cellular senescence and promotes fibroblast-led cancer cell invasion. FEBS Open Bio. 2017 Sep 11;7(10):1586-1597.
17) Han L, Luo H, Huang W et al., Modulation of the EMT/MET Process by E-Cadherin in Airway Epithelia Stress Injury. Biomolecules. 2021 Apr 30;11(5):669.
18) Liang C, Zhao J, Lu J et al., Development and Characterization of MDR1 (Mdr1a/b) CRISPR/Cas9

Knockout Rat Model. Drug Metab Dispos. 2019 Feb;47(2):71-79.

19) Brinkmann U. Functional polymorphisms of the human multidrug resistance (MDR1) gene: correlation with P glycoprotein expression and activity in vivo. Novartis Found Symp. 2002;243:207-10; discussion 210-2, 231-5.
20) Cai QC, Liao H, Lin SX et al., High expression of tumor-infiltrating macrophages correlates with poor prognosis in patients with diffuse large B-cell lymphoma. Med Oncol. 2012 Dec;29(4):2317-22.
21) Zhang J, Yan Y, Yang Y et al., High Infiltration of Tumor-Associated Macrophages Influences Poor Prognosis in Human Gastric Cancer Patients, Associates With the Phenomenon of EMT. Medicine (Baltimore). 2016 Feb;95(6):e2636.
22) Jordaan G, Liao W, Sharma S. E-cadherin gene re-expression in chronic lymphocytic leukemia cells by HDAC inhibitors. BMC Cancer. 2013 Feb 25;13:88.
23) Maxwell SA, Cherry EM, Bayless KJ. Akt, 14-3-3ζ, and vimentin mediate a drug-resistant invasive phenotype in diffuse large B-cell lymphoma. Leuk Lymphoma. 2011 May;52(5):849-64.
24) Julien S, Puig I, Caretti E et al., Activation of NF-kappaB by Akt upregulates Snail expression and induces epithelium mesenchyme transition. Oncogene. 2007 Nov 22;26(53):7445-56.
25) Yagi K, Yamamoto K, Umeda S et al., Expression of multidrug resistance 1 gene in B-cell lymphomas: association with follicular dendritic cells. Histopathology. 2013 Feb;62(3):414-20.
26) Lemma S, Karihtala P, Haapasaari KM et al., Biological roles and prognostic values of the epithelial-mesenchymal transition-mediating transcription factors Twist, ZEB1 and Slug in diffuse large B-cell lymphoma. Histopathology. 2013 Jan;62(2):326-33.
27) Zheng H, Shen M, Zha YL et al., PKD1 phosphorylation-dependent degradation of SNAIL by SCF-FBXO11 regulates epithelial-mesenchymal transition and metastasis. Cancer Cell. 2014 Sep 8;26(3):358-373.

Institutional Review Board: This study was conducted after getting approval of the Ethical Review Committee (ERC-88- 2022) (ERC Jinnah Hospital) and Institutional Review Board (IRB-398/05-2022) of Forman Christian College (A Chartered University).

Conflict of Interest: None

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