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BCL-2 expression level among in CML Patients in Ile Ife

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Abstract
BCL-2 was the first anti-death gene. A key for oncogenic mechanism in several haematological malignancies, has been attributed to the upregulation of BCL-2 which is caused by numerous mechanisms. The aim of the study is to evaluate the BCL-2 mutation by its expression level among haematologic malignant patients in Ile Ife. This study was cross-sectional and a total of 100 consenting participants were recruited: 50 known CML patients and 50 control subjects. Obafemi Awolowo University Teaching Hospital Complex, Ile-Ife, a Referral Centre for haematologic malignancies, and Seventh Day Adventist Hospital, Ile-Ife, were used as the study centers. Full blood count was done using 3-part haematology auto analyzer (Mindray), while RNA extraction and qPCR Bcl-2 quantification were done using real time PCR equipment. The results obtained from the study shows that there are higher expression levels of the Bcl-2 gene in CML samples compared with control samples, attaining significant $p<0.05$. Bcl-2 overexpression, suggests that dysregulated Bcl-2 is potentially involved in the pathogenesis of CML. Using gene expression analysis will prove a powerful tool in the diagnosis, prognosis and evaluation of the diseases and aid in the BCL-2 therapy project.
***Keywords**: BCL-2 expression, CML, leukaemia*

Introduction

B Cell Lymphoma-2 (BCL-2) is one of the Bcl-2 protein family encoded by the BCL-2 gene.[1] It was the first anti-death gene, anti-apoptotic modulator dis-covered to be associated with cancer.[2]

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(BCL-2) gene is located on chromosome 18q21.33. It was first discovered by cloning the breakpoint region of the t (14;18) translocation, a chromosomal abnormality commonly found in follicular lymphomas (FL).[3] Bcl-2 resides within the mitochondria, the cell's powerhouse. The BCL-2 protein is the founding member of the BCL-2 family of apoptosis regulators. It functions by neutralizing pro-apoptotic proteins like Bax and Bak, preventing them from triggering the release of cytochrome c, a crucial step in the intrinsic apoptotic pathway. Mimetics (BH3-only proteins) circumvent BCL-2 and BCL-XL, sequester and inhibit it, however, freeing BAX and BAK to initiate the cascade caspase leading to cell death.[4] This delicate balance between pro- and anti-apoptotic protein maintains cellular homeostasis. However, when Bcl-2 expression surpasses a certain threshold, it disrupts this balance, enabling cell survival and hindering apoptosis.[5-6] So BCL-2 gene expression level/overexpression can be used as a marker to assess BCL-2 gene mutation in the incidence of haematologic malignancies

Materials and Methods

Study Area

The study was carried out in Obafemi Awolowo University Teaching Hospitals Complex (OAUTHC), Ile-Ife, and Seventh Day Adventist Hospital (SDAH), Ile-Ife, Osun State, South-West, Nigeria. Ile-Ife is about 218 kms northeast of Lagos, 40km from Osogbo. Ife is Latitude 7^0 28'N and 7^0 45'N and longitudes 4^0 30'E and 4^0 34'E., east of the city of Ibadan. It has a population of 501,000 people (Sina Ojuade, 1992; Egu, 2011). OAUTHC is along Ilesa road while SDA is at Idiomo in between Mayfair and Lagere, Ile-Ife. (Ife central local government).

Study Design

The study is a descriptive cross-sectional study representing a random sampling of the haematological malignant patients in Ife metropolis. The research is designed to assess the BCL-2 mutation in haematologic malignant patients in Ile-Ife, and was carried out within three months.

Study Population and Subjects

The participants were known Haematological malignant- Chronic myelogenous leukemic (CML) patients who attend the referral haematology clinic and the control group.

Sample size determination

Using formula validated by Adebola *et al.*[7] the sample size was determined as follows

$n = Z2pq/d^2$

(P = prevalence, Q=1-P, d – degree of accuracy (0.05), Z = (1.96), P=0.22, d=0.05, confidence level: 95%). According to Otu and Ejike, 2021, the prevalence of Haematologic Malignancies in Abuja was 6.66%.

P= prevalence of Haematologic malignancies - 6.66% [8]

Then the formular will be considered: Z= 1.96 (for 95% confidence level) d= 0.05 (confidence interval or tolerance error)

N= Sample size $N = \frac{Z2P\ (1-P)}{d^2}$

$= \frac{1.96 \times 2 \times 0.067(1-0.067)}{}$

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$$= \frac{3.92 \times 0.067 \times 0.933}{0.0025} \qquad = 98.02$$

Therefore, the minimum sample size-N is pegged at 100.

Ethical considerations

Ethical clearance was obtained from the review board of Seventh Day Adventist Hospital (SDAH), Ile-Ife, Osun state (SERC-2024-3-0035). Informed consent was obtained from all participants and confidentiality of patient data strictly maintained.

Blood samples collection, storage and transportation

About 4.0ml venous blood samples was collected, 2ml was dispensed into two anticoagulated (EDTA- Ethylene–diamine tetra acetic acid) sample bottles each, i.e 2 sample bottles containing 2ml of blood each for each participant (test and control). One blood sample was meant for full blood count while the second was for RNA extraction and qPCR analysis. Samples were well mixed after collection and labelled appropriately. 200 blood samples were collected in total. From CML patients, 50 blood samples was used for full Blood Count, blood samples were analysed within 8 hours of collection using "auto analyzer machine (3 parts) while another 50 blood samples were kept for RNA extraction and qPCR analysis at -4^0C to prevent haemolysis until sample size (100) was realized. The same was done for the control. The blood sample size-100(50 CML + 50 CONTROL) were transported in cold chain (2 to 8^0C) to Biorepository and Clinical Virology Laboratory, College of Medicine, University of Ibadan for RNA extraction and eventual RT-PCR analysis for BCL-2 gene expression.

Full Blood Count Using (Mindray) Haematology Auto Analyzer

Procedure: Blood samples were placed on blood mixer machine. One after the other, the blood sample was put under the probe of the autoanalyzer. Auto analysis was done for the following parameters: PCV, Total and Absolute WBCs count, Platelet count, and Red Cell indices.

Rna Extraction Protocols (Qiagen Kit)

Arranged in a plastic rack was 2.0 ml Eppendorf tube according to the number of the expected samples to be processed. 560ul of the prepared AVL buffer containing 5.6ul of carrier RNA (probe) was dispensed into the arranged and labelled Eppendorf tube. Into each of the Eppendorf tube, dispensed was 140ul of the fresh or frozen blood samples into corresponding labelled tube. Each sample was vortex for 10secs and spun down briefly using the Microcentrifuge and incubated at room temperature for 10mins. 560ul of absolute ethanol (96% -100%) was added into each labelled tube containing lysed samples (lysis buffer + sample + carrier RNA) (inactivation). Each sample was vortex for 10secs and spun down briefly using the Microcentrifuge placed in the biosafety cabinet 630ul of each lysate (sample + lysis buffer + ethanol) was aspirated into corresponding labelled spin column, and centrifuged for 1min at 12000rpm. Collection tubes

discarded and each spin column was placed into a new collection tube. The remaining 630ul of each lysate (sample + lysis buffer + ethanol) was aspirated into corresponding labelled spin column and centrifuged for 1min at 12000rpm. Collection tubes was discarded and each spin column was placed into a new collection tube. 500ul of WASH buffer AW2 was dispensed into each spin column containing the collection tube and centrifuged for 1min at 12000rpm (9000 x g). Collection tubes containing waste was discarded and each spin column was placed into a new collection tube and centrifuged at 14000rpm (10500 x g) for 3mins. The spin column was placed into a new micro-centrifuge tube(1.5ml) and 60ul of AVE elution buffer was dispensed into each tube containing the spin column Then Incubated at room temperature for 1.0min and centrifuged for 1.0min at 12000rpm. The spin column was discarded

Real Time Polymerase Chain Reaction (Rt-PCR)

Procedure Of One Step Rt-PCR

Primer design

In order to design specific qPCR primers specific to the quantification of Homo sapien BCL-2 genes, Homo sapien BCL-2 were downloaded from the NCBI website (National Center for Biotechnology Information) and multiple cluster analysis was performed to reveal the conserved regions. Homo sapien BCL-2 genes of accession number NM_000633.3 was used as a DNA reference sequence and used in the primer designing. https://www.idtdna.com/PrimerQuest/Home/Index site was accessed and sequence pasted in the sequence entry box and multiple intercalating dye PCR primers were generated. It is very necessary to ensure that the primers will have a perfect match, this will enhance primer annealing during PCR. To do this, primers must anneal to regions where the sequences are conserved. Each primer pair was then checked for specificity to be sensitive to only the genes of interest to which it was designed to detect and also ability to cut across all aligned genes then the best primer was selected and synthesized at Inqaba in South Africa.

RNA treatment

20 ng total RNA was then treated with NEB DNase 1 (M0303) to totally eliminate extracted DNA briefly, a mixture of 2µl of 10ng/ µl RNA, 10 µl DNase I Reaction Buffer (10X), 1 µl DNAse I (RNase-free) and up to 100 µl with Nuclease-free H_2O.the mixture was then Incubated at 37°C for 10 minutes followed by Adding 1 µl of 0.5 M EDTA (to a final concentration of 5 mM). then Heat inactivated at 75°C for 10 minutes and stored in the -20^{o}C till use

Gene quantification

A volume of 20 µl reactions following manufacturer's instructions using Luna® Universal qPCR Master Mix Protocol (M3003) was used to detect the presence of miRNA genes in the extracted RNA. Expression of Actin gene was used as an internal control. Briefly, A mix of 10 µl Luna Universal qPCR Master Mix, 0.5 µl Forward primer (10 µM) 0.5 µl Reverse primer (10 µM) and 0.06 Reverse Transcriptase (Promega) made up to 18 µl with Nuclease-free Water to which 2 µl of the treated RNA Template was added. This was then ran with the profile Initial Denaturation 95°C for 60 seconds followed by 40-45 of Denaturation 95°C 15 seconds Extension and plate

reading at 60°C for 30 seconds followed by a termination at 72ºC for 10 minutes. Amplification was conducted using the cfx96tm real time system from bio-rad following manufacturer manual.
One-Step RT-PCR: It is a type of RT – PCR where the reverse transcription and the amplification reactions occur in a single tube. All the required components are added in a single tube. First, reverse transcription occurs, forming cDNA, which is then amplified in a PCR process.
Luna Universal qPCR Master Mix and other reaction components was thawed at room temperature, then placed on ice. After thawing completely, each component was mixed briefly by inversion, pipetting or gentle vortexing.
The total volume for the appropriate number of reactions was determined, plus 10% overage and assay mix of all components was prepared except DNA template accordingly. Then it was mixed thoroughly but gently by vortexing. Liquid collected to the bottom of the tube by brief centrifugation. Aliquot Assay mix was aliquoted into qPCR tubes or plate. For best results, ensure accurate and consistent pipetting, volumes and minimized bubbles was ensured. DNA templates was added to qPCR tubes or plate. Tubes were sealed with flat optically transparent caps, and plates sealed with optically transparent film. To prevent artifacts caused by evaporation, care was taken to have plate edges and corners sealed properly. Tubes or plates was spun briefly to remove bubbles and collect liquid (1minute at 2500 – 3000rpm). Real – time instrument was programmed with indicated thermocycling protocol. It was ensured that a "plate read" is included at the end of the extension step. Used was the STBR or SYBR/FAM scan mode setting on the real-time instrument or "Fast" cycling profile RT phase at 42 ^{O}C for 30 minutes, the Promega (RT) converts RNA to cDNA. Data was analyzed according to real – time instrument manufacturer instructions.

Statistical analysis

The collected data was cleaned, coded, summarized, and checked for accuracy, consistency and completeness. The data is carefully entered into the Statistical Package for social science (SPSS IBM version 22) statistical software and analyzed using descriptive statistics such as mean, median, mode, standard deviation and bivariant analysis with T-test and cross tabulation/ correlation analysis with bar chat and histograms.

Results

In figure 4.1, there is comparison of BCL-2 expression between control and test. In this study, it was discovered that the BCL-2 overexpression in the Test (CML patients) is 20(40%) which is higher than that of the Control (non-cml) subject which is 5(10%). The Bcl-2 with no expression: the Test is 30(60%) lower than that of the control which is 45(90%).

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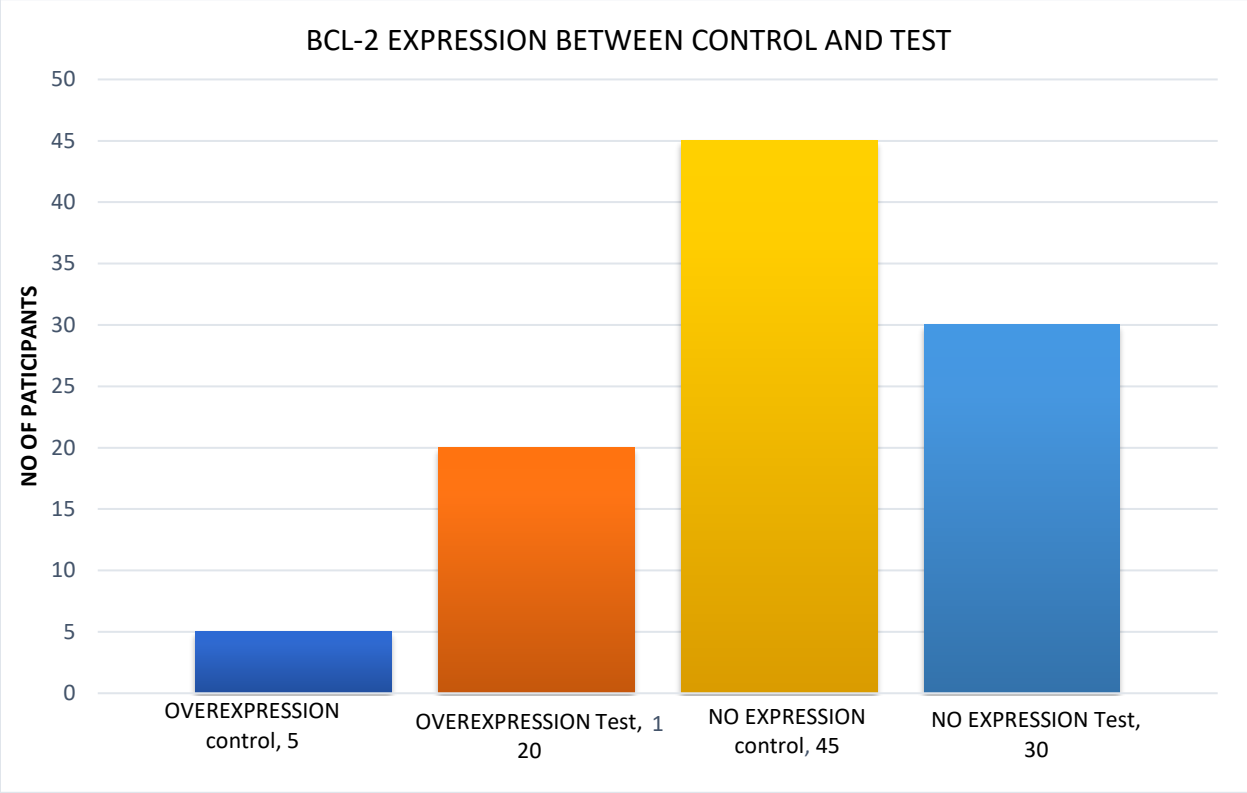

Figure 1: BCL-2 EXPRESSION COMPARED BETWEEN CONTROL AND TEST

In figure 2, BCL2 gene expression in CML patients in relation WBC count is figured out. In this study, among the 40% CML with BCL-2 overexpression,15(30%) has leukocytosis >12.0 – 450.0 x 109/L and 5(10%) has normal WBCs count range from 3.0 – 12.0 x 109/L. Among the 60% CML with no expression, 29(58%) has normal WBCs count while 1(2%) has low WBC <3.0 x 109/L.

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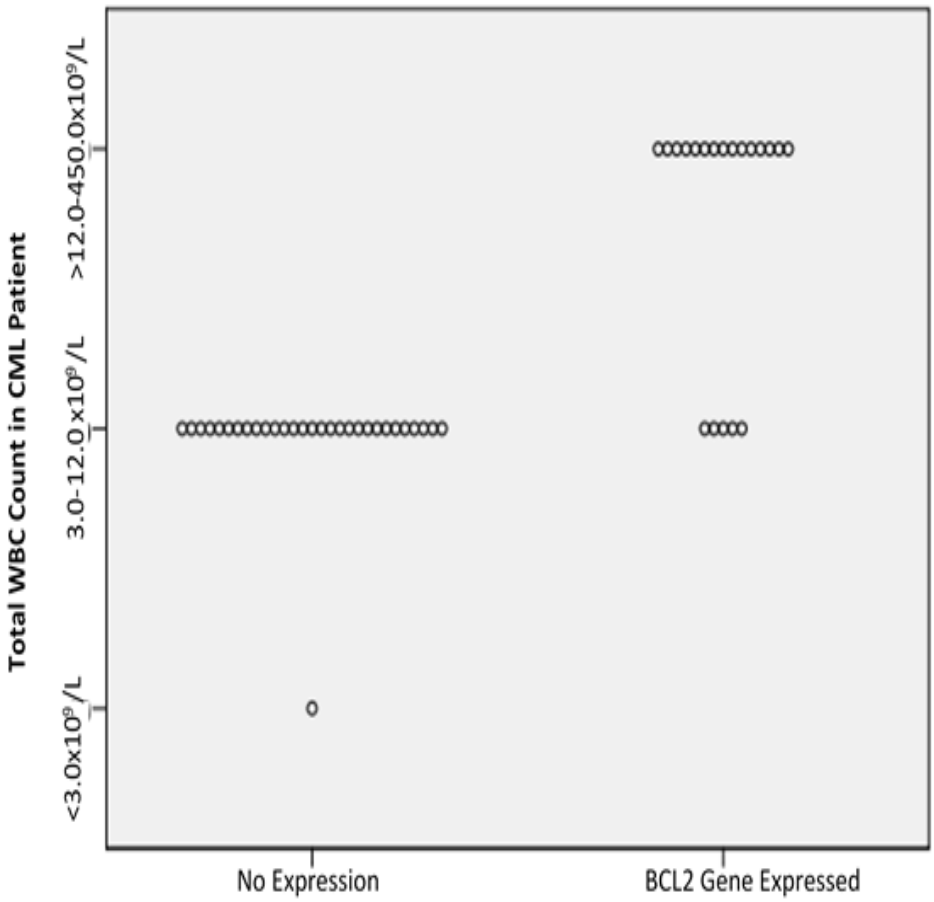

Figure 2: Bcl-2 gene expression in CML patients in relation to WBCs count (P=0.00)

The relationship between BCL2 gene expression and WBC in **control subject** is examined in fig.4.5. In this study, 43(86%) control subjects out of 50 has WBC within the normal reference range 3.0 – 12.0 x 109/L, 43(86%) were BCL-2 of no expression and 5(10%) were BCL-2 of overexpression. 2(4%) with no expression of BCL-2 has low WBC < 3.0 x 109/L.

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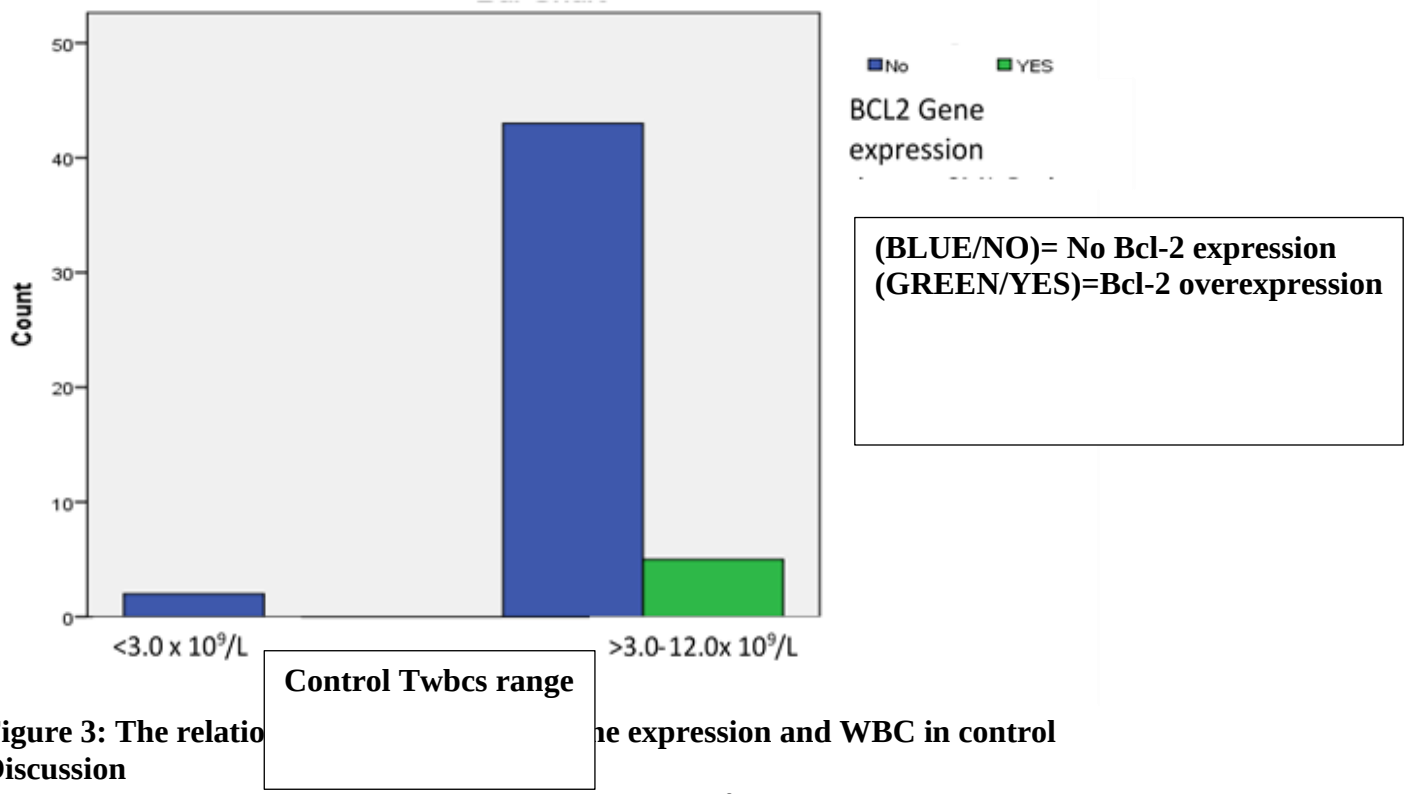

Figure 3: The relatio ne expression and WBC in control

Discussion

The delicate balance between pro- and anti-apoptotic forces maintains cellular homeostasis, however, when Bcl-2 expression surpasses a certain threshold, it disrupts this balance.[5-6] CML is associated with increased Bcl-2 expression at the protein and mRNA levels.[9] Bcl-2 expression level is a marker for the role of Bcl-2 gene in the incidence of disease.[1]

The findings in this study observed 40% BCL-2 overexpression among the CML patients compared to 10% BCL-2 overexpression among the control subjects attaining significant P< 0.05. It was also noted that (90%) control subjects show no expression which further support the findings of this study suggesting that the pathogenesis of CML is possibly due to the involvement of BCL-2 overexpression. This finding is in correlation with the study by Melo *et al*. [9] that CML is associated with increased BCL-2 expression at the protein and mRNA.

Findings noted among the 40% CML patients with BCL-2 overexpression shows that 14% have normal Packed cell volume (PCV), 24% has lower PCV while 2% has higher PCV. The study shows that out of the 40% CML patients with gene overexpression, 24% has lower PCV. It was also noted among the 60% CML patients with no expression: 16% had lower PCV while 44% has normal PCV. This implies that more than halve of CML with overexpression have anaemia while more than halve of those with no expression have normal PCV attaining significance P < 0.05.

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This study found that the 40% CML patients that showed BCL-2 overexpression, 30% has leukocytosis, 10% has normal WBC count. It was also noted that 60% CML patients with no expression of BCL-2, 58% has normal WBC count while 2% has leukopenia. Study by Meto *et. al.* [9] posited that CML is associated with increased BCL-2 expression, leading to uncontrolled proliferation of leukemic cells.

Conclusion

The overexpression observed in this study is suggestive of, a level that is characteristic of CML patients, and its association with anaemia, leukocytosis and neutrophilia. The potential of Bcl-2 overexpression as a diagnostic marker for hematologic malignancies stems from its frequent association with the diseases. Bcl-2 overexpression holds promise as a diagnostic marker for specific hematologic malignancies like MM and particularly FL with the t (14;18) translocation.

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