

Bone Marrow Profile in Haematological Disorders with reference to Flow Cytometry and RT-PCR in Acute Leukaemia

BINDHUJA JOSEPH¹, SS MEGA SAMLY², LIJI ANNA VARGHESE³

ABSTRACT

Introduction: Evaluation of non neoplastic and neoplastic haematological disorders require bone marrow examination which is an important diagnostic tool. This includes Bone Marrow Aspiration (BMA) and Bone Marrow Biopsy (BMB). Subtyping of Acute Leukaemia (AL) requires flow cytometry immunophenotyping and Real Time Polymerase Chain Reaction (RT-PCR), which helps in identifying cell antigens and genetic abnormalities, respectively. This is helpful to guide specific treatment for patients.

Aim: To evaluate the clinical profile, cytological and histological pattern of various haematological disorders using bone marrow examination and to determine immunophenotypes using ancillary techniques in patients with AL.

Materials and Methods: This was a cross-sectional observational study conducted in Department of Pathology, Sree Mookambika Institute of Medical Sciences, Kulasekharam, Tamil Nadu, India. Data was collected for a period of two years from May 2020 to April 2022. A total of 62 cases were included. Clinical details and bone marrow examination findings were noted for all BMA and BMB cases that satisfied the inclusion criteria and flow cytometry along with RT-PCR diagnosis was done for suspected AL cases.

Analysis was done using Statistical Package for the Social Sciences (SPSS) version 20.0.

Results: Among the 62 cases studied, age of patients ranged from 32-81 years. Majority of them were in the 5th to 6th decade. Females 32 (51.6%) were more commonly affected. Pancytopenia 15 (24.2%) was the most common clinical presentation. Total 49 (79%) were diagnosed with BMA and 61 (98.4%) were diagnosed with BMB. Megaloblastic anaemia 16 (25.8%) and acute myeloid leukaemias 6 (9.6%) were the most common cause of benign haematological disorder and haematological malignancy, respectively. The RT-PCR test for Break point Cluster-Abelson Tyrosine Kinase (BCR-ABL) and Promyelocytic Leukaemia-Retinoic Acid Receptor Alpha (PML-RARA) fusion gene analysis showed association in patients with Acute Lymphoblastic Leukaemia (ALL) and Acute Myeloid Leukaemia (AML), respectively.

Conclusion: Biopsy, being gold standard, provides details about the pattern, extent of tumour, metastatic deposit and granulomatous pathology, but BMA also proved better for study of the cell. Flow cytometry and RT-PCR were effective tools that enable the identification of immunophenotype in AL as well as to assess treatment progress and predict prognosis.

Keywords: Anaemia, Bone marrow aspiration, Bone marrow biopsy, Cluster of differentiation, Pancytopenia

INTRODUCTION

Haematological disorders are quite frequent in all age group and they range from anaemias, infectious disorders to malignancies. Bone Marrow Examination (BME) has an important role in diagnosis of non neoplastic and neoplastic diseases and assessing patient prognosis [1]. The BME plays a crucial role in bone marrow transplantation. Two important techniques used are BMA and BMB. Both are done simultaneously for accurate evaluation of bone marrow [2]. A BMA is useful in making out better individual cell morphology. It is well suited for examination by cytogenetics, Polymerase Chain Reaction (PCR) and Flow Cytometric methods. Whereas BMB is useful in bone marrow pattern, distribution, inadequate or failed aspiration, focal lesions like granulomatous diseases, bone marrow fibrosis, staging of lymphomas and metastasis [3]. Examination of bone marrow needs clinical details, complete blood count, peripheral blood smear, special stain and immunohistochemistry to diagnose AL, lymphomas and to grade marrow fibrosis in chronic myeloproliferative neoplasms [4].

Acute leukaemia is a highly heterogeneous malignant disease, and it is divided into two major types: AML and ALL. The AML is subclassified into eight subtypes (AML M0-M7) and ALL is subclassified into three subtypes (ALL L1-L3) based on morphology, immunological and molecular genetics. Cytogenetic analysis using World Health Organisation (WHO) classification is the gold standard for assessment of AL [5].

The subtyping of AL is an important step for correct treatment and prognosis. Flow cytometry immunophenotyping identifies cell membrane antigens using "Clusters of Differentiation" (CD) markers and fluorochromes that are applied to cell suspension. This also helps to detect minimal residual disease. With many advanced techniques, detection of fusion genes was important for the diagnosis, classification, and prognosis of AL [6]. This study was aimed to evaluate the clinical profile, cytological and histological pattern of various haematological disorders using BME and to determine the immunophenotypes of acute leukaemia using ancillary techniques like flow cytometry and RT-PCR. Objectives of the present study was to diagnose diseases affecting bone marrow using BMA and BMB, to evaluate the expression of immunemarkers in subtyping ALs and to identify BCR ABL and PML RARA fusion using RT-PCR for treatment and prognosis. Flow cytometry and RT-PCR helps to identify immunophenotype of AL and to find the clinical usefulness of these different phenotypes in prognosis of patients with AL.

MATERIALS AND METHODS

This was a cross-sectional observational study carried out in Department of Pathology, Sree Mookambika Institute of Medical Sciences, Kulasekharam, Tamil Nadu, India, conducted over a period of two years from May 2020 to April 2022.

Ethical clearance was obtained (IHEC No:52/2019). Informed consent was obtained from all patients before performing BMA and biopsy.

Based on the study conducted by Fareed N et al., [7], prevalence of haematological disorder was found to be 79.7%.

Sample size calculation: Sample size was calculated using the formula $n = Z_{1-\alpha/2} \cdot pq/d^2 = 62.15 = 62$ cases

where, $p = 79.7\%$

$q = 20.3\%$

$d = 10\%$ absolute error

$Z_{1-\alpha/2} = 1.96$ for $\alpha = 5\%$

Inclusion criteria: All cases undergoing bone marrow studies where both aspiration and biopsy was done were included in the study.

Exclusion criteria: Cases where only aspiration was available and follow-up cases were excluded from the study.

Study Procedure

Clinical details were documented. An Ethylenediaminetetraacetic Acid (EDTA) blood sample was collected from all patients for complete blood count and peripheral smear examination. Smears was prepared for all cases and stained with Leishman stain. The BMA and BMB was done under aseptic precautions from posterior iliac spine. The procedure was done under local anaesthesia (Inj. 2% xylocaine). For aspiration and biopsy, LP 16 G needle and Jamshidi needle were used, respectively. Aspiration sample was collected for performing BMA smears, flow cytometry and RT-PCR. A BMA smear was prepared and stained with Giemsa and Perl's stain. The BMB sample was received in Bouins fixative, after grossing it was processed in alcohol and xylene. Embedded in paraffin wax, sections were cut in 3-4 μ thickness, stained with Haematoxylin and eosin (H&E) stain. Smears and biopsy slides were viewed and diagnosis was reached. In cases of AL, flow cytometry and RT-PCR were done.

For flow cytometry a panel of monoclonal antibodies (Immunotech) were used, that included myeloid markers (MPO, CD13, CD14, CD15, CD33, CD117, and CD11b), T-cell lineage markers (CD2, CD3, cyCD3, CD5, and CD7), B-cell lineage markers (CD10, CD19, and CD20), megakaryocytic lineage markers (CD41 and CD61) and other markers (CD34, CD38, HLA-DR, CD71, CD9, CD25, CD103, CD56, Anti KAPPA and Anti LAMBDA). Comprehensive antibody with direct immunofluorescent labelling was used for immunophenotyping with a FACSCanto II System and Diva software. The blast population was gated on the basis of light scattering properties. Unstained cells in each tube were used as negative controls to set quadrant gates. The BCR ABL and PML RARA fusion gene analysis was done using RT quantitative PCR kit - HG-U133 plus2 (Affymetrix). Each probe included FAM (6-carboxy-fluorescein; emission, 518 nm) at the 5'-end as the reporter and TAMRA (6-carboxy-tetramethylrhodamine; emission, 582 nm) at the 3'-end as the quencher. The primer and probe sequences were designed using Primer Express software.

Non probability sampling technique with all consecutive cases that satisfied the inclusion criteria were included in this study (62 cases). The BMA slides were examined for the cellularity, myeloid erythroid ratio, erythroid series, myeloid series, lymphoid series, plasma cells, histiocytes, megakaryocytes and abnormal cell infiltration. In addition to the findings mentioned in BMA, BMB slides were viewed for pattern of infiltration, granulomas, Abnormal Infiltration of Immature Precursors (ALIP) and marrow fibrosis. In flow cytometry percentage of antigen positive cells (cut-off $>20\%$) was calculated using the quadrant statistics. Expression of one or more markers on more than 20% of the blast cell population was considered. Based on the positivity of immune markers subtyping of acute leukaemia was done. The BCR p190 copy number, BCR p210 copy number, ABL copy number and PML RARA copy numbers were assessed and type of transcript was detected in RT-PCR.

STATISTICAL ANALYSIS

Data entered in excel sheet and analysis was done using SPSS 20.0 software. Frequency of haematological disorder was presented as

percentage and 95% confidence interval. The association between haematological disorder and demographic variable was tested for statistical significance using Chi-square test. A p-value of less than 0.05 was considered as statistically significant.

RESULTS

A total of 62 cases were included in this study. Patients age ranged from 32-81 years. Majority of the cases 15 (24.2%) cases were in 5th to 6th decade. Females 32 (51.6%) were more commonly affected than males 30 (48.4%).

Pancytopenia 15 (24.2%) and anaemia 12 (19.4%) were the most common clinical indications for performing bone marrow examination. Distribution of indications for bone marrow examination is depicted in [Table/Fig-1].

Indications	Frequency	Percent %
Pancytopenia	15	24.2
Anaemia	12	19.4
Pyrexia of unknown origin	2	3.2
Splenomegaly	6	9.7
Hepatosplenomegaly	7	10.5
Lymphadenopathy	2	3.2
Suspicious of multiple myeloma	9	14.5
Thrombocytosis	1	1.6
Thrombocytopenia	2	3.2
Metastatic work up	6	9.7
Total	62	100

[Table/Fig-1]: Distribution of cases according to indication of bone marrow examination.

Association between age, gender and BMB diagnosis was tested and no association was found, since the p-values were 0.3 and 0.4, respectively [Table/Fig-2,3].

Sensitivity, specificity Positive Predictive Value (PPV) and Negative Predictive Value (NPV) of BMB was 98.33%, 100%, 100% and 66.66%, respectively and in BMA was 76.99%, 100%, 100% and 20%, respectively.

In BMB, nutritional anaemia includes both iron deficiency and megaloblastic anaemia (16 cases, 25.8%) was the most common benign haematological disorder and acute leukaemia (12 cases, 19.3%) was the most common malignant haematological disorder in this study [Table/Fig-4]. Among the 62 cases, 49 (79%) were diagnosed with BMA and 98.4% (61 cases) were diagnosed with BMB. Total 61 (98.4%) were diagnosed by both BMA and BMB while 1 (1.62%) was inconclusive because of inadequate sampling technique. The concordance rate and inconclusive cases with final haematological diagnosis (N=62) is described in [Table/Fig-4].

Of the remaining 12 cases that were inconclusive in BMA, 1(1.6%) case was diagnosed as megaloblastic anaemia, 1(1.6%) as iron deficiency anaemia, 1(1.6%) as immune thrombocytopenic purpura and 1(1.6%) as plasma cell dyscrasia [Table/Fig-5,6] in BMB. The BMA in these cases were diluted. About 2 (3.2%) and 4 (6.4%) cases showed myeloproliferative neoplasm [Table/Fig-7,8] and metastatic deposit [Table/Fig-9,10] in BMB, respectively. Marrow fibrosis/packed marrow due to infiltration by neoplastic cells can yield hypocellular aspirate. This highlights the importance of trephine biopsy. About 2 (3.2%) cases that had no diagnosis in BMA, showed granulomas in BMB [Table/Fig-11,12]. Fibrosis in and around granuloma may cause difficult marrow aspiration.

Twelve cases of acute leukaemia required other ancillary investigations like flow cytometry and RT-PCR. Flow cytometry was done for subtyping based on WHO classification [6]. Flow cytometry uses different immune phenotypic markers that help to differentiate subtypes of AML and ALL [Table/Fig-13]. Flow cytometry of B ALL and leukaemic phase of mantle cell lymphoma was shown in [Table/Fig-14,15], respectively.

Age (years)	Acute leukaemia	Myeloproliferative neoplasms	Lymphoproliferative neoplasm	Plasma cell dyscrasia	Iron deficiency anaemia	Megaloblastic anaemia	Metastasis	Myelodysplastic syndrome	Granulomatous lesion	Normal study	Immune thrombocytopenic purpura	Aplastic anaemia	Inconclusive	Total	p-value
31-40	2	2	0	1	1	7	0	0	0	0	0	0	0	13	0.3
41-50	3	3	0	2	3	0	0	0	1	1	1	0	0	14	
51-60	4	2	1	2	1	0	3	0	0	1	0	1	0	15	
61-70	2	1	0	3	1	0	2	1	1	0	0	1	1	13	
71-80	1	0	0	0	1	2	1	1	0	0	0	0	0	6	
81-90	0	0	0	1	0	0	0	0	0	0	0	0	0	1	
Total	12	8	1	9	7	9	6	2	2	2	1	2	1	62	

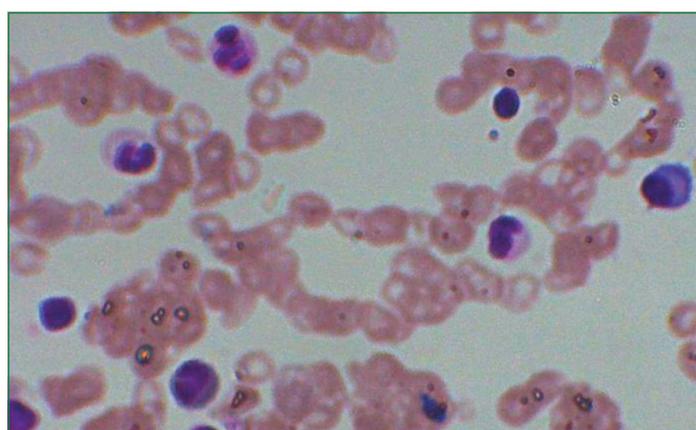
[Table/Fig-2]: BMB diagnosis based on age distribution.

Parameters	Acute leukaemia	Myeloproliferative neoplasms	Lymphoproliferative neoplasm	Plasma cell dyscrasia	Iron deficiency anaemia	Megaloblastic anaemia	Metastasis	Myelodysplastic syndrome	Granulomatous lesion	Normal study	Immune thrombocytopenic purpura	Aplastic anaemia	Inconclusive	Total	p-value
Male	4	4	1	3	1	6	3	1	2	1	1	2	1	30	0.4
Female	8	4	0	6	6	3	3	1	0	1	0	0	0	32	
Total	12	8	1	9	7	9	6	2	2	2	1	2	1	62	

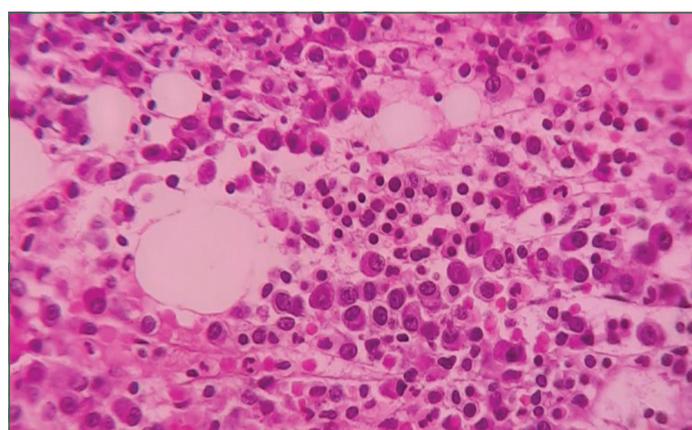
[Table/Fig-3]: BMB diagnosis based on gender distribution.

Diagnosis	BMB		BMA		BMB and BMA (concordance)	Inconclusive in BMA but diagnosed with BMB	Inconclusive BMA and BMB
	Frequency	%	Frequency	%			
Acute leukaemia	12	19.3	12	19.3	12 (100%)	0	0
Myeloproliferative neoplasms	8	12.9	6	9.7	6 (75%)	2 (25%)	0
Lymphoproliferative neoplasm	1	1.6	1	1.6	1 (100%)	0	0
Plasma cell dyscrasia	9	14.5	8	12.9	8 (89%)	1 (11%)	0
Iron deficiency anaemia	7	11.3	6	9.7	6 (86%)	1 (14%)	0
Megaloblastic anaemia	9	14.5	8	12.9	8 (89%)	1 (11%)	0
Metastasis	6	9.7	2	3.2	2 (33%)	4 (67%)	0
Myelodysplastic syndrome	2	3.2	2	3.2	2 (100%)	0	0
Granulomatous lesion	2	3.2	0	0	0	2 (100%)	0
Normal marrow study	2	3.2	2	3.2	2 (100%)	0	0
Immune thrombocytopenic purpura	1	1.6	0	0	0	1 (100%)	0
Aplastic anaemia	2	3.2	2	3.2	2 (100%)	0	0
Inconclusive	1	1.6	13	21.1	0	0	1 (100%)
Total	62	100	62	100	49	12	1

[Table/Fig-4]: Comparison of BMB and BMA diagnosis of haematological disorders.



[Table/Fig-5]: Case-1: Diluted BMA with rare plasma cell (Leishman stain 40X).



[Table/Fig-6]: Case-1: BMB plasma cell dyscrasia- multiple myeloma (H&E 40X).

spatial distribution and extent of infiltrates and overall cellularity. This is comparable to study done by Puri V et al., [14]. Among the 9 (14.5%) cases of plasma cell dyscrasias, 7(11.6%) cases were of multiple myeloma that was comparable to study done by Reshma ST et al., [15] who reported that incidence of plasma cell dyscrasia was 9.57%.

The ITP was diagnosed in 1 case (1.6%) in this study which was comparable to study conducted by Bashir N et al., [11] and Reshma ST et al., [15] who reported 1.3% and 0.87%, respectively. Total 8 (12.9%) cases were diagnosed as myeloproliferative neoplasms in BMB. In CML cases, aspirates were better able to classify the phases as compared to biopsy. Two cases of granulomatous pathology were diagnosed on BMB. Aspirate showed reactive marrow similar to study done by Gilotra M et al., [16]. About 49 (79%) and 61 (98.4%) cases in this study were diagnosed with BMA and biopsy, respectively. Gilotra M et al., [16] in their study diagnosed 87% cases with BMA and the remaining required biopsy for correct diagnosis. Total 49 (80.33%) cases showed concordance in both BMA and BMB, and was comparable to study conducted by Gilotra M et al., [16] with 72.4% concordance in BMA and BMB.

In this study sensitivity for diagnosing haematological disorders was high for BMB (98.33%) when compared to BMA (76.99%), so BMB was considered as gold standard. This is similar to Goyal S et al., [17] who showed a sensitivity of 88.5%. Among the 12 AL cases diagnosed on BMA and biopsy, flow cytometry found 4(6.4%) B-ALL, 1 (1.6%)T-ALL, 2 (3.2%) AML M2, 3(4.8%) AML M3, 1(1.6%) AML M4 and 1 (1.6%) leukaemic phase of mantle cell lymphoma. Large number of cells can be examined quickly by flow cytometry, which improves the accuracy of leukaemia diagnosis and helps in detection of mixed phenotypic AL [18].

In the present study, all patients with AML M3 were BCR-ABL p190 and p210 negative, whereas four patients with ALL were BCR-ABL p190 positive and p210. The AML-M3 cases showed PML positivity. A study revealed that patients of AL who exhibit BCR-ABL mutations seemed to have a worse prognosis than those without these mutations [19]. This states that detection of fusion gene was important for the diagnosis, classification, and prognosis of AL [20].

Limitation(s)

Touch imprints were not evaluated in study which may have increased the diagnostic accuracy. Analysis of cytogenetic immunophenotype correlations was not performed due to the small sample size in this study. A larger sample size is needed to explore this association in future studies.

CONCLUSION(S)

This study concluded that BMA and BMB complimented each other and the superiority of one method over the other depended on the underlying disorder. Both procedures are used routinely nowadays and do not require sophisticated equipment's. Hence BMA and BMB should be done simultaneously as they play important role

for making diagnosis of haematological disorders. Further, flow cytometry and RT-PCR analysis provides rapid, and effective method to aid in diagnosis, treatment prognosis and to predict prognosis in patients with AL.

REFERENCES

- [1] Tietsche de MoraesHungria V, Chiattono C, Pavlovsky M, Abenzoza LM, Agreda GP, Armenta J, et al. Epidemiology of hematologic malignancies in real-world settings: Findings from the Hemato-oncology Latin America observational registry study. *Journal of Global Oncology*. 2019;5:01-09.
- [2] Chauhan S, Pradhan S, Mohanty R, Saini A, Devi K, Sahu MC, et al. Evaluation of sensitivity and specificity of bone marrow trephine biopsy tests in an Indian teaching hospital. *Alexandria Journal of Medicine*. 2018;54(2):161-66.
- [3] Khan MI, Rasheed A, Iqbal S. Bone marrow aspiration versus trephine biopsy: Diagnostic value of both modalities when done simultaneously. *The Professional Medical Journal*. 2021;28(12):1844-49.
- [4] Lagoo AS, Rosenthal NS. Bone marrow at initial diagnosis: Clinical associations and approach to diagnosis. *In Practical Lymph Node and Bone Marrow Pathology* 2020;447-64.
- [5] Khoury JD, Solary E, Abla O, Akkari Y, Alaggio R, Apperley JF, et al. The 5th edition of the World Health Organization classification of haematolymphoid tumours: Myeloid and Histiocytic/Dendritic neoplasms. *Leukemia*. 2022:01-07.
- [6] Kruse A, Abdel-Aziz N, Kim HN, Ruan Y, Phan V, Ogana H, et al. Minimal residual disease detection in acute lymphoblastic leukemia. *International Journal of Molecular Sciences*. 2020;21(3):1054.
- [7] Fareed N, Mehmood T, Quraishy MS, Fatima G. Pattern of hematological disorders on bone marrow examination: A tertiary care hospital experience. *Hematol Transfus Int J*. 2021;9(6):117-20.
- [8] Bain BJ, Clark DM, Wilkins BS. *Bone marrow pathology*. John Wiley & Sons; 2019.
- [9] Joshi-Warpe S, Wader J, Warpe BM. Bone marrow aspiration and bone marrow biopsy in hematological disorders. *Biomedical and Pharmacology Journal*. 2020;13(2):799-04.
- [10] Ranabhat S, Maharjan S, Tiwari M, Bhandari A, Osti BP. Bone marrow aspiration cytology in the diagnosis of hematologic and non-hematologic diseases in a multi-specialty hospital in Nepal. *Int J Res Med Sci*. 2017;5(3):922-26.
- [11] Bashir N, Musharaf B, Reshi R, Jeelani T, Rafiq D, Angmo D, et al. Bone marrow profile in hematological disorders: An experience from a tertiary care centre. *Int J Adv Med*. 2018;5(3):608-13.
- [12] Alwahaibi NY, Al-Himali S, Kumar JS. Capability of hematoxylin and eosin stain to demonstrate hemosiderin in bone marrow trephine biopsy. *J Cytol Histol*. 2012;3:144.
- [13] Vijayamohan L, Asotra S, Kumari K, Murgai P, Dattal D. Comparative utility of bone marrow aspiration and trephine biopsy in evaluation of hematological disorders. *Archives of Medicine and Health Sciences*. 2020;8(1):15.
- [14] Puri V, Sharma P, Kotru M, Sikka M, Sharma S. Utility of simultaneous assessment of bone marrow aspirates and trephine biopsy sections in various haematological disorders. *Iraqi Journal of Hematology*. 2018;7(1):26.
- [15] Reshma ST, Haque S, Chowdhury ZZ, Kabir MI, Rabhani MG, Rabby MA, et al. Spectrum of haematological & non haematological disorder diagnosed by bone marrow examination: A study of 115 cases. *Medicine Today*. 2022;34(1):07-11.
- [16] Gilotra M, Gupta M, Singh S, Sen R. Comparison of bone marrow aspiration cytology with bone marrow trephine biopsy histopathology: An observational study. *Journal of Laboratory Physicians*. 2017 c0078;9(03):182-89.
- [17] Goyal S, Singh UR, Rusia U. Comparative evaluation of bone marrow aspirate with trephine biopsy in hematological disorders and determination of optimum trephine length in lymphoma infiltration. *Mediterr J Hematol Infect Dis*. 2014;6(1):e2014002.
- [18] Porwit A, Béné MC. Multiparameter flow cytometry applications in the diagnosis of mixed phenotype acute leukemia. *Cytometry Part B: Clinical Cytometry*. 2019;96(3):183-94.
- [19] Kang ZJ, Liu YF, Xu LZ, Long ZJ, Huang D, Yang Y, et al. The Philadelphia chromosome in leukemogenesis. *Chinese Journal of Cancer*. 2016;35(1):01-05.
- [20] Chen X, Wang F, Zhang Y, Ma X, Cao P, Yuan L, et al. Fusion gene map of acute leukemia revealed by transcriptome sequencing of a consecutive cohort of 1000 cases in a single center. *Blood Cancer Journal*. 2021;11(6):1-0.

PARTICULARS OF CONTRIBUTORS:

1. Associate Professor, Department of Pathology, Sree Mookambika Institute of Medical Sciences, Kulasekharam, Tamil Nadu, India.
2. Assistant Professor, Department of Pathology, Sree Mookambika Institute of Medical Sciences, Kulasekharam, Tamil Nadu, India.
3. Statistician Cum Tutor, Department of Community Medicine, Sree Mookambika Institute of Medical Sciences, Kulasekharam, Tamil Nadu, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:

Bindhuja Joseph,
119, Jose Bhavan, Puthukudiyerupu, Nagercoil, Tamil Nadu, India.
E-mail: bindhuja85@gmail.com

PLAGIARISM CHECKING METHODS: [Jain H et al.]

- Plagiarism X-checker: Jun 16, 2022
- Manual Googling: Aug 20, 2022
- iThenticate Software: Sep 10, 2022 (19%)

ETYMOLOGY: Author Origin

AUTHOR DECLARATION:

- Financial or Other Competing Interests: None
- Was Ethics Committee Approval obtained for this study? Yes
- Was informed consent obtained from the subjects involved in the study? Yes
- For any images presented appropriate consent has been obtained from the subjects. Yes

Date of Submission: **Jun 07, 2022**

Date of Peer Review: **Jul 22, 2022**

Date of Acceptance: **Sep 12, 2022**

Date of Publishing: **Jan 01, 2023**