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Original research article

Expression of apoptosis and proliferation markers in acute leukemias by immunohistochemistry

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Abstract

Aim: To assess the level of expression of the apoptotic markers survivin, p53, Bcl-2 and proliferation marker MIB-1 in acute leukaemia's by IHC in bone marrow biopsies.

Methodology: It was a Descriptive cross-sectional study. The study was carried out over a one year period from 01 Jan 2022 to 01 Jan 2023. The study covered all acute leukaemia cases encountered over a 1 years period retrospectively and prospectively from 01 Jan 2022 to 01 Jan 2023. All cases of acute leukaemia who underwent bone marrow biopsy as a part of clinical evaluation.

Results: In our study of 40 consecutive cases of AL, most of our cases were AML type (75%). We found statistically significant relation between apoptosis markers like p53 and Bcl-2; p53, Bcl-2 & survivin in AL cases. Acute Leukaemia's are highly aggressive hematologic malignancies. The genesis and progression of the leukemic disease invariably involve deregulation in the apoptotic response pathway. Targeted therapies that are designed to induce apoptosis in leukemic cells are currently the most promising antileukemia strategies. We evaluated the relation between survivin expression (IHCn) and p53 mutation (both with anti-apoptotic functions) in AL cases along with the correlation of nuclear staining with other measured parameters.

Conclusion: The understanding of the molecular apoptotic machinery and of its defects in AL lays the basis for developing new treatment strategies like drugs able to induce apoptosis of leukaemia cells.

Keywords: Leukemia, Bcl2, p52, Survivin, bone marrow biopsies

Introduction

Leukaemias are neoplastic proliferations of hematopoietic cells. Specific genetic events contribute to malignant transformation of cells and their progeny forming a clone of leukaemic cells. Acute leukaemias are defined as 'neoplasms with more than 20% blasts in the peripheral blood/bone marrow (WHO)'. There are two main groups of acute leukaemias: Acute Myeloid Leukaemia (AML) and Acute Lymphoblstic leukaemia (ALL).

Acute leukaemias comprise a large number of leukaemias which differ in aetiology, pathogenesis, morphology, course and prognosis. With recent advances in molecular biology and treatment modalities, it is essential to subtype the leukaemia to assess the prognosis and institute a specific chemotherapy. The two classifications presently in use are:

a) FAB classification.
b) WHO classification (2008).

FAB classification is based on morphology and cytochemistry. In the FAB classification ALL is divided into 3 subtypes (L1-L3), AML is divided into 8 subtypes (M0-M7).

The WHO classification separates ALL into Precursor B/precursor T lymphoblastic leukemia/lymphoma and AML into 7 subgroups based on morphologic, genetic, immunophenotypic features. In addition to morphology and cytochemical evidence of lineage, flow cytometry is used to classify acute leukaemias based on the presence of myeloid and lymphoid antigens.

A common component of leukaemogenesis and drug resistance in acute leukaemias is resistance to apoptosis. Apoptosis occurs by two pathways, both of which result in caspase activation:

a) Receptor-mediated, involving the tumor necrosis factor (TNF) family of death receptors.
b) Mitochondrial-mediated, regulated by the Bcl-2 family of proteins.

Overexpression of Bcl-2 and other anti-apoptotic proteins, including Bcl-X(L), XIAP, p53 and survivin is documented in solid tumours. It is important to study and evaluate apoptosis dysregulation and pathways in acute leukaemias. The study will help in better understanding of tumour biology and will lead to devising better targeted strategies to manage the disease in the future.

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IHC is a robust and standard technique used in the lab to evaluate presence or absence of a target protein. In addition it also gives a visual location of the presence and concentration of target protein in the cell of interest. IHC will be done using survivin, p53, Bcl-2, MIB-10.

Survivin
Survivin is a member of the inhibitor of apoptosis (IAP) family. The survivin protein functions to inhibit caspase activation, thereby leading to negative regulation of apoptosis or programmed cell death. This has been shown by disruption of survivin induction pathways leading to increase in apoptosis and decrease in tumour growth.

p53
Tumor protein p53, also known as p53 is a protein that in humans is encoded by the TP53 gene. It can initiate apoptosis-programmed cell death-if DNA damage proves to be irreparable. p53 is the most frequently inactivated protein in human cancer, and more than 50% of all solid tumors carry a mutation in the *TP53* gene that abrogates its DNA binding and transactivation activity. Since the inactivation of p53 in cancer has been associated with poor survival, refractory disease, and chemo resistance, p53 gene therapy and reactivation of mutant p53 have been designed to restore p53 function.

Bcl-2
Bcl-2 (B-cell lymphoma 2), encoded in humans by the *BCL2* gene, is the founding member of the Bcl-2 family of regulator proteins that regulate cell death (apoptosis), by either inducing it (pro-apoptotic) or inhibiting it (anti-apoptotic). Bcl-2 is specifically considered as an important anti-apoptotic protein and is thus classified as an oncogene. Antibodies to Bcl-2 can be used with immunohistochemistry to identify cells containing the antigen.

MIB-1
MIB-1 is an antibody directed at the protein Ki-67 and a product of the MKI67 gene. MIB-1 is used in clinical applications to determine the *Ki-67 labelling index*. The Ki-67 protein is a cellular marker for proliferation.
Thus, we plan to study these markers in cases of acute leukaemias.

Aim and Objectives
Aim
To assess the level of expression of the apoptotic markers survivin, p53, Bcl-2 and proliferation marker MIB-1 in acute leukaemia's by IHC in bone marrow biopsies.

Objectives
i) To study expression of apoptosis by immunohistochemistry using survivin, p53, Bcl-2 in Acute leukemia's.
ii) To study expression of proliferation by using MIB-1 IHC in Acute leukemia's.
iii) To study the correlation of apoptosis and proliferation markers with subtype of acute leukaemia's.

Material and Methods
Study design
Descriptive cross-sectional study.

Place of study
Department of Pathology of a Medical College and tertiary care centre.

Duration of study
The study was carried out over a one year period from 01 Jan 2022 to 01 Jan 2023. The study covered all acute leukaemia cases encountered over a 1 years period retrospectively and prospectively from 01 Jan 2022 to 01 Jan 2023.

Inclusion criteria
All cases of acute leukaemia who underwent bone marrow biopsy as a part of clinical evaluation.

Exclusion criteria
Cases of acute leukaemia where diagnosis based on Peripheral blood smear and bone marrow aspirate was done.
All cases diagnosed as "Acute leukaemias" except those mentioned under exclusion criteria, in tertiary care centre were included. A minimum of 40 acute leukaemias were studied. Cases were taken both retrospectively (where bone marrow biopsy was available) and prospectively. Suitable controls were

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taken both for staining technique as well as biological control.

Histomorphological study
All slides were reevaluated and histomorphological features noted. The present study was based on histomorphological and immunohistochemical evaluation of acute leukaemia cases. Age, demographic characteristics, clinical and biochemical findings were also recorded in prospective cases. For retrospective cases, data was taken from records. Survival data and disease free interval with molecular cytogenetics results available for 21 AML cases.

Blocks and Staining
Paraffin blocks of all cases which qualified for the inclusion criteria (both prospective and retrospective) were obtained and 3-5 µm tissue sections were cut and stained with hematoxylin and eosin (H&E) stain. Special stains (IHC) were used as required. They were evaluated by two pathologists.

Histopathological studies
Bone marrow biopsy tissues were fixed in Formalin and Paraffin embedded. Slides were prepared from these Formalin Fixed Paraffin Embedded specimens. Slides were stained by Haematoxylin and Eosin.
Review of Haematoxylin and Eosin-stained slides were done to ascertain the acute Leukaemia subtype according to FAB classification.

Immunohistochemical studies
Survivin, p53, Bcl-2, MIB-1 Antibody detection were carried out on FFPE sections by IHC (Annexure D).
The antibodies used in this study were:
For p53: Monoclonal Mouse anti-human, clone DO-7, Ready to use, LOT AM2390126 (Biogenex).
For Bcl-2: Monoclonal Mouse anti-human, clone 124, Ready to use, LOT 1112205F (Cell Marque).
For Survivin: Monoclonal Rabbit anti-human, Ready to use, EP2880Y, Biogenex.
For MIB-1: Monoclonal Mouse anti-human Ki-67 antigen, clone MIB-1, Isotype IgG1 kappa, Ready to use, LOT 00095712 (Dako).

Interpretation of IHC results
For P53, Bcl-2 and survivin, cases were scored on a three-tiered system, with 0-24% of blast cells staining called negative, 25-75% positivity scored as 1 and 76-100% scored as 2. Each stain was given a score on a scale of 0 to 2 to evaluate the intensity. (0-Negative, 1-weak interrupted patchy staining, 2-diffuse strong positivity). At least 200 cells were counted in each case visually. Suitable controls were taken both for staining as well as biological control (P53, Bcl-2-Breast carcinoma; Survivin-Bladder carcinoma; MIB-1-High grade lymphoma). Finally index retrieved from multiplication of percentage of positive blasts with intensity score. Only brown coloured nuclear staining was regarded as positive for P53, MIB 1 and only cytoplasmic staining considered positive for Bcl-2. Survivin protein expression was considered positive if there was either cytoplasmic or nuclear envelope staining. For MIB 1, cases were scored as <1%, 2-5% and >6% positive blasts [1].

Statistics
Statistical analysis was done using IBM SPSS (Statistical package for Social Sciences) statistics software version 20. Analysis of various markers with type and risk category of leukaemia was done using chi-square tests. Less than 0.05 was taken as significant for p value.

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Observations and results

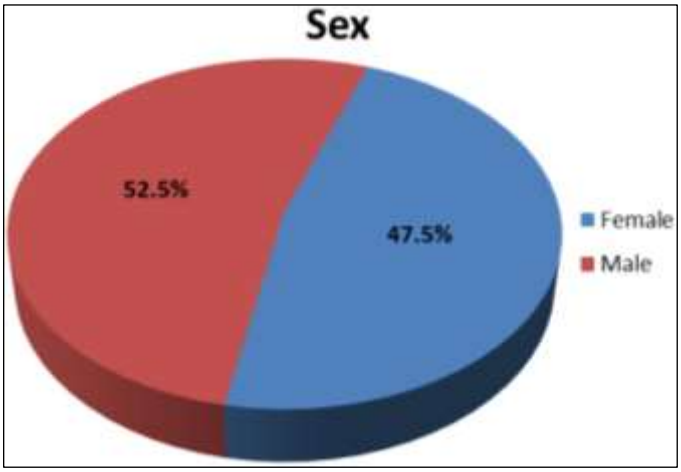

Fig 1: Showing distribution sex in 40 acute leukaemia cases

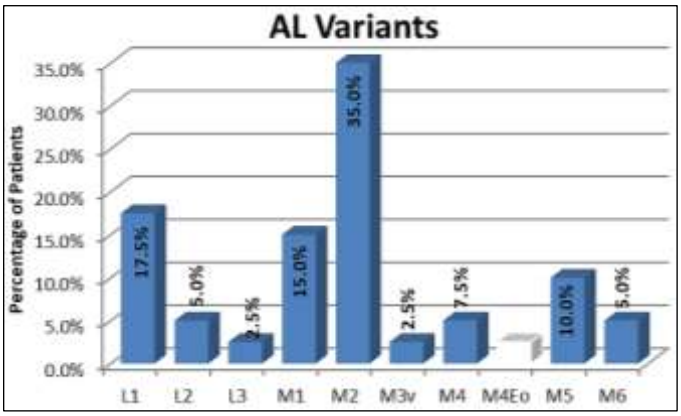

Fig 2: Showing distribution of acute leukaemia cases according to FAB classification

Table 1: Shows frequency distribution of demographic and clinical data of ALL and AML patients included in the study variables

Frequency distribution of demographic and clinical data of ALL and AML patients included in the study Variables	All patients N (%)	AML patients N (%)
Hepatomegaly		
Negative	3 (30)	4 (13)
Positive	7 (70)	26 (87)
Splenomegaly		
Negative	4 (40)	6 (20)
Positive	6 (60)	24 (80)
Lymphadenopathy		
Negative	6 (60)	24 (80)
Positive	4 (40)	6 (20)
Extramedullary involvement: CNS manifestations		
Negative	10 (100)	30 (100)
Positive	0 (0.0)	0 (0.0)
Testicular enlargement		
Negative	8 (80)	-
Positive	2 (20)	-

N, number of cases; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CNS, central nervous system.

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P53

Table 2: Shows intensity of P53 expression in acute leukaemia cases included in the study variables

	Frequency	Percentage
0	8	20.0%
1.0	10	25.0%
2.0	22	55.0%
Total	40	100.0%

Table 3: Shows P53 expression in percentage of blasts in acute leukaemia cases included in the study variables

	Frequency	Percent
0	8	20.0%
1.0	10	25.0%
2.0	22	55.0%
Total	40	100.0%

Table 4: Shows P53 index in acute leukaemia cases included in the study variables

	Frequency	Percent
0	8	20.0%
1.0	7	17.5%
2.0	6	15.0%
4.0	19	47.5%
Total	40	100.0%

Table 5: Shows intensity of Bcl-2 expression in acute leukaemia cases included in the study variables

	Frequency	Percent
0	4	10.0%
1.0	15	37.5%
2.0	21	52.5%
Total	40	100.0%

Table 6: Shows Bcl-2 expression in percentage of blasts in acute leukaemia cases included in the study variables

	Frequency	Percent
0	4	10.0%
1.0	15	37.5%
2.0	21	52.5%
Total	40	100.0%

Table 7: Shows Bcl-2 index in acute leukaemia cases included in the study variables

	Frequency	Percent
0	4	10.0%
1.0	5	12.5%
2.0	20	50.0%
4.0	11	27.5%
Total	40	100.0%

Survivin

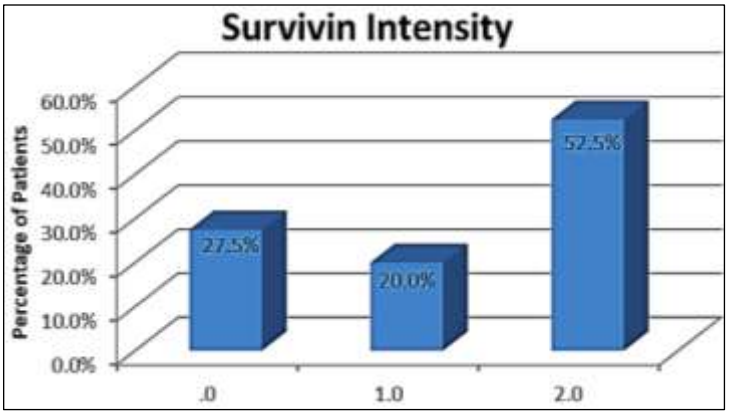

Fig 3: Shows intensity of Survivin expression in acute leukaemia cases included in the study variables

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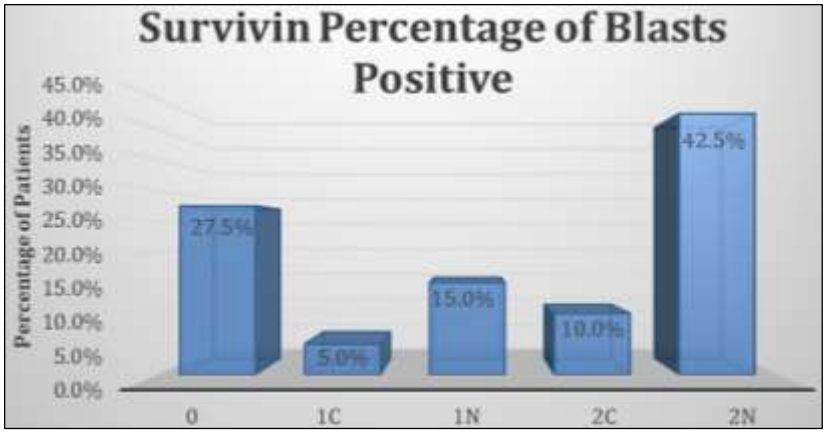

Fig 4: Shows Survivin expression in percentage of blasts in acute leukaemia cases included in the study variables C, Cytoplasmic; N, Nuclear

Table 8: Shows Survivin index in acute leukaemia cases included in the study variables

	Frequency	Percent
0	11	27.5%
1.0	2	5.0%
2.0	13	32.5%
4.0	14	35.0%
Total	40	100.0%

Table 9: Shows MIB-1 scoring in acute leukaemia cases included in the study variables

	Frequency	Percent
<1%	8	20%
2-5%	11	27.5%
>6%	21	52.5%
Total	40	100.0%

Intensity comparison

Table 10: Shows intensity comparison between P53, Bcl-2 and Survivin in acute leukaemia cases included in the study variables

Intensity	P53		Bcl2		Survivin	
	Frequency	Percentage	Frequency	Percentage	Frequency	Percentage
0	8	20.0%	4	10.0%	11	27.5%
1.0	10	25.0%	15	37.5%	8	20.0%
2.0	22	55.0%	21	52.5%	21	52.5%
Total	40	100.0%	40	100.0%	40	100.0%

Table 11: Demonstrates impact of antibodies p53, Bcl 2, survivin and MIB 1 in acute leukaemiatypes. N, number of cases; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia

Case (N)	P53 Index	Bcl 2 index	Survivin index	MIB 1 scoring
AML M1 (6)	4	4	4	>5%
AML M2 (14)	2	2	2	1-5%
AML M3 (1)	4	4	4	>5%
AML M4 (2)	4	2	2	1-5%
AML M5 (4)	2	2	2	<1%
AML M6 (2)	4	4	4	>5%
ALL L1 (7)	2	2	2	1-5%
ALL L2 (2)	4	4	4	>5%
ALL L3 (1)	4	4	4	>5%

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Risk stratification with survivin antibody in 21 AML cases based on cytogenetics, molecular genetics

Table 12: Showing risk stratification with survivin antibody in 21 AML cases based on cytogenetics, molecular genetics

Risk category (Number of cases 'n')	Survivin index		Survival duration (in months)	P-Value
	Nuclear	Cytoplasmic		
Favourable (n=6)	2 (n=2)	2 (n=4)	42	P = 0.4141
Intermediate (n=9)	4 (n=4)	2 (n=5)	30.5	P = 0.7387
Adverse (n=5)	4 (n=4)	4 (n=1)	6.5	P = 0.1797

Table 13: Demonstrates impact of survivin in AML cases according to whether it is demonstrated by IHC in nucleus (IHCn), by IHC in cytoplasm (IHCc) along with other markers. Nuclear survivin expression present in the majority of the AL cases we studies, did correlate with poor prognostic factors (unfavourable subtype, mutant p53, poor cytogenetics)

	AML with Flt3 and 7q-	AML with NPM1 and CEBPA	AML with Normal cytogenetics
Number of cases	5	6	9
Average survival duration in months	2	8	18
Survivin index	4	2	2
Survivin nuclear positivity	4/4	2/5	4/9
Survivin cytoplasmic positivity	1 / 4	4/6	5/9
P53 Index	4	2	2
Bcl2 Index	4	2	2
MIB-1 >5%	9/10	2/6	7/11

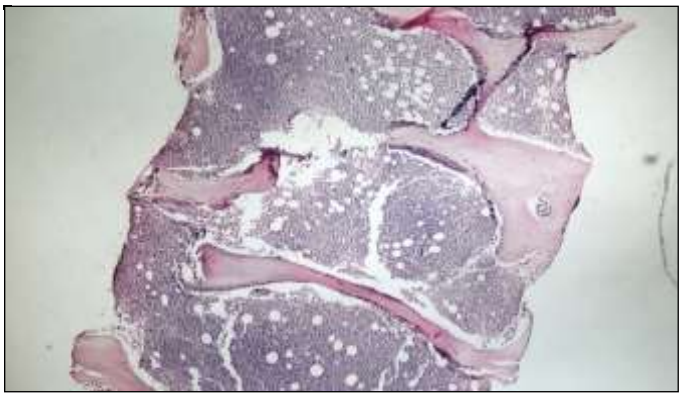

Fig 5: Bone marrow showing complete replacement by blasts (H&E, × 40)

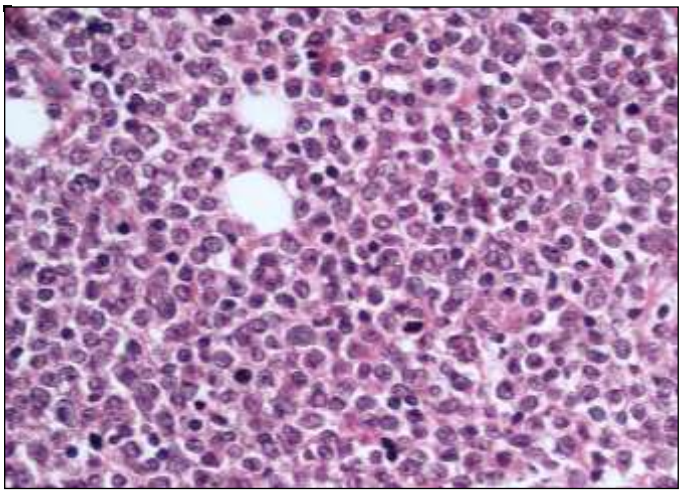

Fig 6: Bone marrow showing predominant blast population (H&E, × 400)

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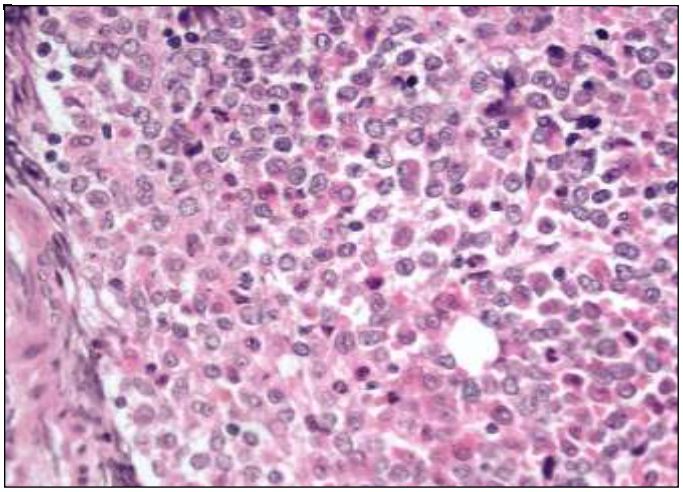

Fig 7: Bone marrow showing blasts with increased eosinophilic precursors (H&E, × 400)

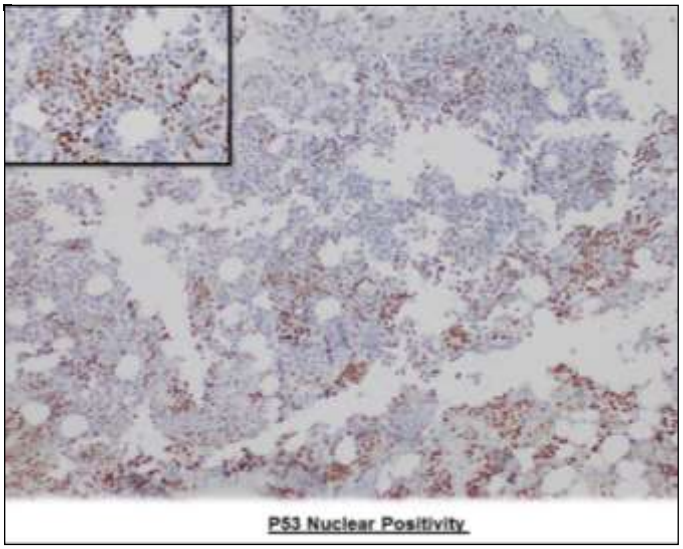

Fig 8: Positive p53 in blasts (p53 IHC, x40); inlet showing p53 diffuse nuclear positive blasts (p53 IHC, 400x)

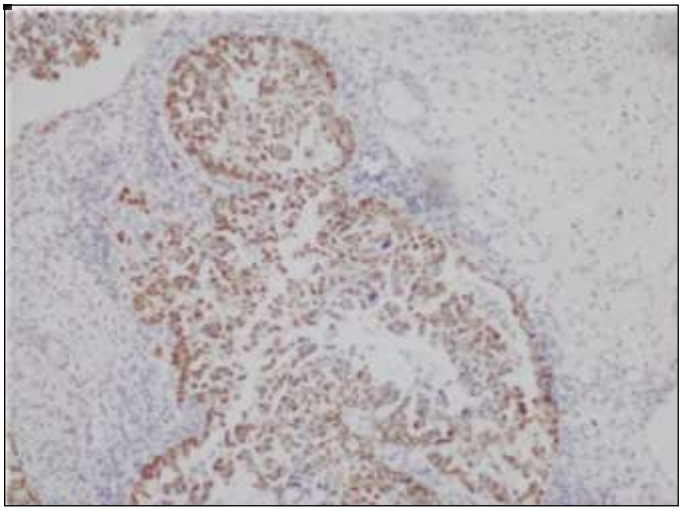

Fig 9: Positive control showing p53 positivity (p53 IHC, x40)

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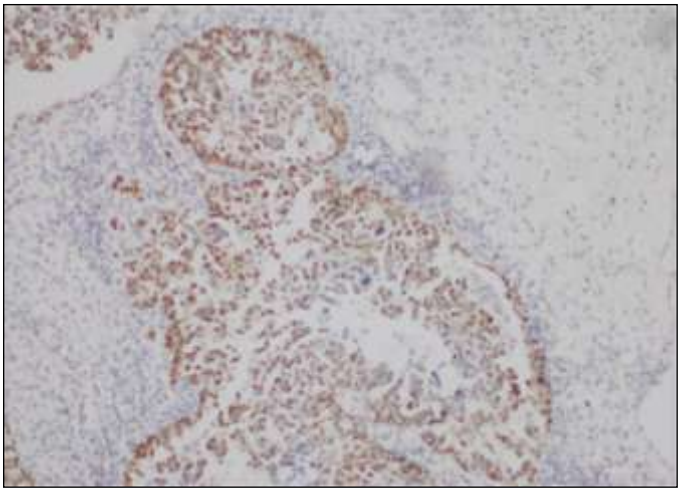

Fig 10: Positive control showing p53 positivity (p53 IHC, x40)

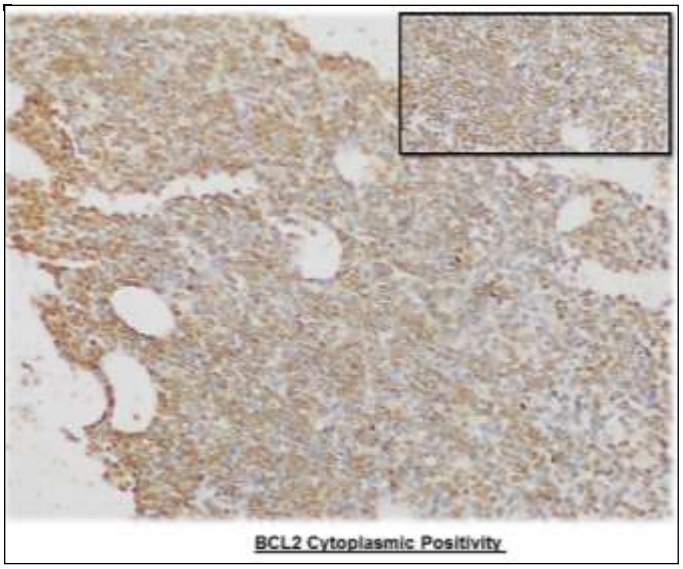

Fig 11: Positive Bcl 2 in blasts (Bcl2 IHC, x40); inlet showing Bcl 2 diffuse cytoplasmic positive blasts (p53 IHC, 400x)

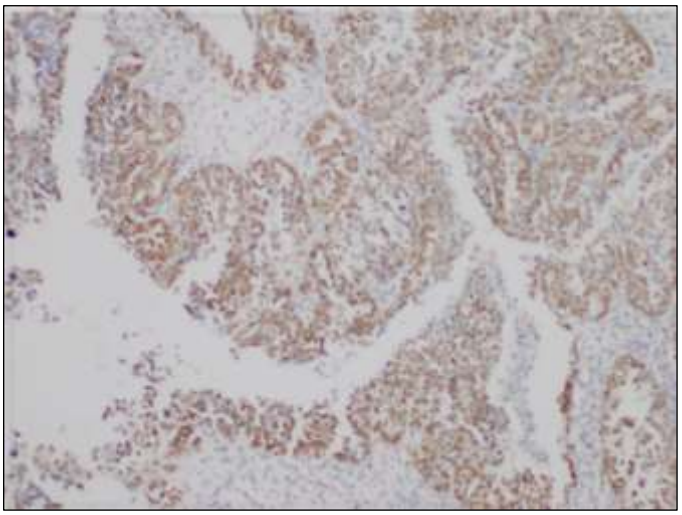

Fig 12: Positive control showing Bcl 2positivity (Bcl 2 IHC, x40)

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Fig 13: Positive survivin in blasts (Survivin IHC, x40); inlet showing surviving nuclear positive blasts (Survivin IHC, 400x)

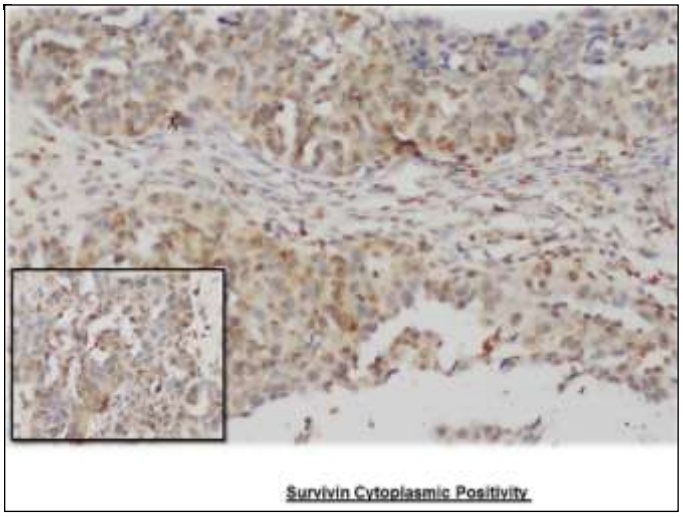

Fig 14: Positive survivin in blasts (Survivin IHC, x40); inlet showing surviving cytoplasmic positive blasts (survivin IHC, 400x)

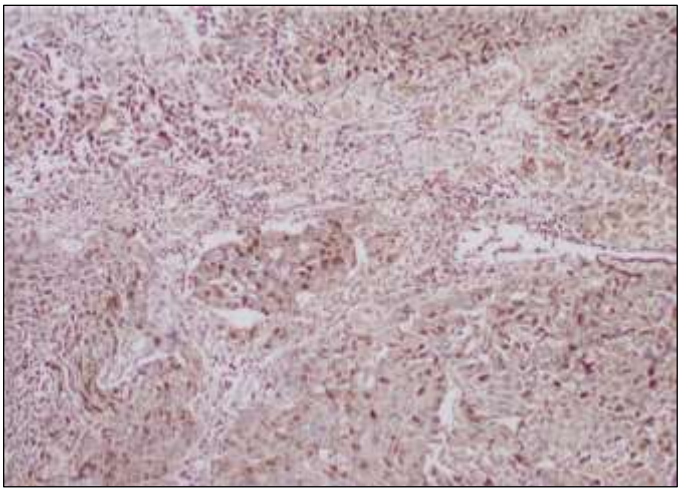

Fig 15: Positive control showing surviving positivity (Survivin IHC, x40)

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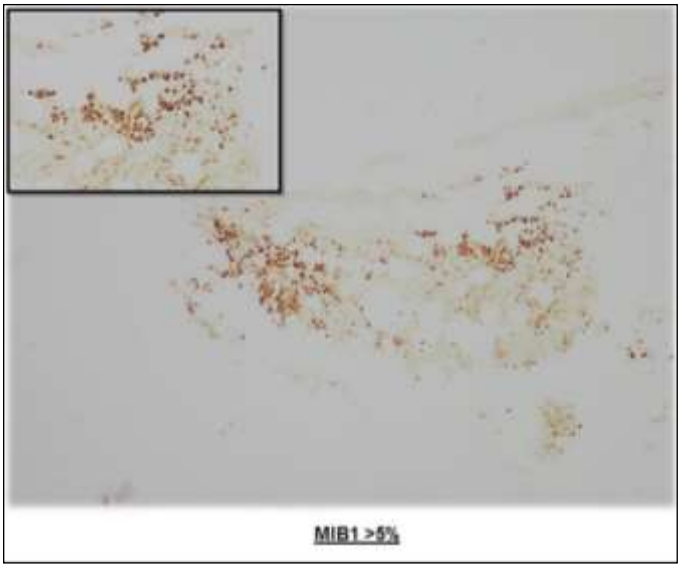

Fig 16: MIB 1 positivity in the blasts (MIB-1 IHC, x40); inlet showing MIB-1 nuclear positivity (MIB-1 IHC, x400)

Discussion

Of the 40 cases of acute leukaemia cases studied in the present study, 30 cases were acute myeloid leukaemia type and 10 cases were acute lymphoblastic leukaemia type. Out of 30 cases of acute myeloid leukaemia type, 6 were M1, 14 were M2, 1 was M3, 3 were M4, 4 were M5 group and 2 were M6 type. Out of 10 cases of acute lymphoblastic type, 7 were L1, 2 were L2 and 1 was L3 type. Immunophenotyping was done on ALL bone marrow biopsies, 8 were B-ALL and 2 were T-ALL. Comparative analysis of incidence was done with various other studies and is shown in Table 14.

Table 14: Comparison of percentage incidence of AML and ALL tumors in different studies and present study

Category	Xie *et al.* [2] (1998)	Ku HG *et al.* [3] (2011)	Present study
AML-M1	13.5	13	15
AML-M2	34.5	38.5	35
AML-M3	2.8	1.5	2.5
AML-M4	6.5	7	7.5
AML-M5	9.5	10.4	10
AML-M6	5.5	4.6	5
ALL-L1	18.5	16.5	17.5
ALL-L2	7	6.4	5
ALL-L3	2.2	2.1	2.5

The major fraction of AL in the present study comprises AML cases (75%), followed by ALL cases (25%).

Distribution of AL according to the age

Acute leukaemia's can occur at any age, even in children and in old age. In the present study mean age of AML cases is 46 (range 34-58) and mean age of ALL cases is 16 (range 3-29) years. Comparative analysis of distribution of cases in various age groups was done with various other studies and is shown in Table 15.

Table 15: Percentage distribution of cases in various age groups in comparison with present study

	Xie *et al.* [2] (1998)	Ku HG *et al.* [3] (2009)	Present study
AML 25-35	12.2	11.6	15.2
AML 36-59	87.8	88.4	84.8
ALL 0-9	53.6	49.5	41.7
ALL 10-29	46.4	50.5	58.3

Others study showed incidence of AML tumours to be more common in 35-59 years of age group. However, our study shows a higher incidence of AML in 40-59 years age group as compared to other studies. However, if the age group of 35-59 is considered the maximum number of cases in all studies are comparable. Age distribution among AML cases seen in the present study was compared with other studies. In the present study, the majority of AML cases occurred in the age group of 40-59 years. This finding is consistent with the studies by Xie *et al.* [2].

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Others study showed incidence of ALL tumours to be more common in 0-15 years of age group. However, our study shows a higher incidence of ALL in 0-19 year's age group as compared to other studies. However, if the age group of 0-20 is considered the maximum number of cases in all studies are comparable. Age distribution among ALL cases seen in the present study compared with other studies. In the present study, the majority of ALL cases occurred in the age group of 0-19 years. This finding is consistent with studies by Xie *et al.* [2].

Distribution of Acute leukemia cases on the basis of FAB classification

40 acute leukaemia cases were classified according to FAB classification. Relative percentage of different types of acute leukaemia cases, compared with other studies is shown in table 14.

Present study results correlated with all other studies which showed AML tumours to be the commonest type. Among the individual AML types, the commonest types were M2 (47%), M1 (20%) followed by M5 (13%), M4 (10%), M6 (7.5%) and M3 (2.5%). Among ALL, L1 type (70%) was the most common followed by L2 (20%) and L3 (10%). Similar findings were seen in studies by Xie *et al.* [2].

In the present study, among the 10 cases of ALL, testicular enlargement was seen in both T-ALL cases (100%). This finding is similar to the study by Ku HG *et al.* [3] who reported testicular involvement in majority of T-ALL cases.

Survivin expression has been demonstrated in solid malignancies and in hematopoietic malignancies [5, 1]. Survivin, as an inhibitor of apoptosis, appears to have a role in cancer progression or drug resistance [1]. Survivin expression may be a useful diagnostic, prognostic, and predictive marker in certain malignancies. The presence of serum anti-survivin antibodies in patients with lung and colorectal cancer may prove a novel diagnostic marker [1].

Studies have shown prognostic impact of survivin in AML cases according to whether it is demonstrated by IHC in nuclei (IHCn), by IHC in cytoplasm (IHCc). Sadek *et al.* showed significant shorter disease-free survival show a significant association with proliferation, clinical stage, histologic subtype, clinical outcome, and survival rate in AML cases with nuclear survivin expression [4]. Several reports have shown nuclear survivin immunostain to have prognostic significance, possibly related to its cell cycle effect, whereas cytoplasmic survivin, with its antiapoptotic effect, has no prognostic significance [5, 1, 6, 7, 8]. Survivin was also found to correlate positively with p21, p27, and p53. A study, in an *in vivo* mouse model, has shown that overexpression of survivin is sufficient to initiate hematologic malignancies, and that hematopoietic cells engineered to overexpress survivin are less susceptible to apoptosis. In our study for survivin extra time for antigen retrieval that is 45 minutes instead of routine 30 minutes used in the process of immunohistochemistry [9].

Immunohistochemical staining revealed a very high frequency of expression in AML cells and relatively high frequency in ALL cells in the present study. Thus, Immunohistochemical analysis would be useful for detecting the few remaining leukaemic cells after treatment and the very early stage of leukaemic relapse of AML/ALL cases on formalin fixed routine bone marrow biopsy samples.

It is difficult to explain the difference in apoptotic character of AML and ALL only by the expression of survivin, p53 and Bcl 2 at this moment. However, it is possible that the ability of survivin to counteract apoptosis is modulated by its localization to the nucleus or the cytoplasm of cell. In addition to its anti-apoptotic function, survivin also plays a role in the regulation of cell cycle progression during mitosis [4, 11].

In contrast to the high frequency of p53 mutations in many of the solid cancers, AML and ALL cases have been shown to demonstrate a high frequency of p53 mutation [4, 11]. We also observed that immunohistochemical accumulation of p53 was present in 90% of cases with AML and 70% of ALL cases. Therefore p53 mutation appear to be the major factor controlling the overexpression of survivin in the bone marrow of AML and ALL cases.

We found that survivin nuclear expression in most of the acute leukaemia cases with adverse prognostic (unfavourable cytogenetics, p53 and Bcl-2 mutation).

Inhibition of apoptosis is a common property of cancer cells, enabling them to increase their survival and facilitate their escape from immune surveillance and cytotoxic therapies. Survivin, as an inhibitor of apoptosis appears to have a role in cancer progression or drug resistance [12, 13].

We show an insignificant correlation between p53 and Bcl-2 expression, P53, Bcl-2 and survivin expression (both with anti-apoptotic functions) and increased proliferation by using MIB 1 in AL cases. Thus, several reports show nuclear survivin immunostain to have prognostic significance, possibly related to its cell cycle effect, whereas cytoplasmic survivin with its antiapoptotic effect, has no prognostic significance [14]. Nevertheless, nuclear survivin expression present in the majority of the AL cases we studies, did compare with poor prognostic factors (unfavourable subtype, mutant p53, poor cytogenetics) and may predict response to antisurvivin therapies that induce apoptosis reduction as a result of survivin expression.

Conclusion

The sample size is small to draw definite conclusions at this stage but the pilot study shows the

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possibility of such a scientific evaluation with larger number of better stratified cases. Nevertheless, nuclear survivin expression, present in the majority of the AL cases we studied, seems to correlate with poor prognostic factors (unfavourable subtype, mutant p53, poor cytogenetics) and may be useful in predicting response to anti-survivin therapies that induce apoptosis as a result of reduced survivin expression. The understanding of the molecular apoptotic machinery and of its defects in AL lays the basis for developing new treatment strategies like drugs able to induce apoptosis of leukaemia cells.

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