Correlation of Morphological and Cytochemical Features of Acute Leukemia

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Original Research Article

ABSTRACT

Acute leukemia is uncontrolled proliferation of the malignant cell i.e. blast cell in the bone marrow and spillage in the peripheral blood also leading to various hematological degenerations as well as systemic deformations by the route of metastasis. There are various methods of diagnosing acute leukemia right from peripheral blood smear examination to molecular laboratory diagnostics. Each method has its own importance. In our study we will be correlating morphological and cytochemical features of acute leukemia which is a cost efficient substitute in our Indian medical system where financial constrains play a major role in treatment.

KEY WORDS: acute leukemia, blast cell, cytochemical stains, AML , ALL.

INTRODUCTION:

Leukemia is a malignant progressive disease in which the bone marrow and other blood forming organs produce increased number of immature or abnormal leucocytes. These suppress the production of normal blood cells, leading to anemia and other symptoms[1].

Acute leukemias are a heterogenous group of malignancies that result from malignant transformation of immature hematopoietic cells followed by clonal proliferation and accumulation of transformed cells. They are characterized by aberrant differentiation and maturation of the malignant cells, with a maturation arrest and accumulation of more than 20% of leukemic blast cells in the bone marrow[2]. There are four important methods of diagnosis in acute leukemia i.e. morphology, cytochemistry, cytogenetics and immunophenotyping. Each one has got its diagnostic and prognostic importance. Acute leukemia is a major health problem in North India with prevalence of all subtypes[3].

We have coupled the morphological features of acute leukemia seen in the peripheral blood film and bone marrow aspirate with the cytochemical stains that is MPO, PAS, NSE and Sudan black in order to get a accurate diagnosis. Proper and effective use of morphological and cytochemical studies of bone marrow aspirate will definitely help out in diagnosing acute leukemia and will reduce the cost to be spent on Immunophenotyping and cytogenetics studies which add a burden to the pocket of the patient[4].

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MATERIALS AND METHODS:

Stained smears prepared for peripheral smear reporting in pathology laboratory were studied and patients with abnormal blood morphology were subjected to detailed examination including history. Presence of abnormal blood morphology like presence of blast cells, presence of dysplastic WBC cells, leucocytosis were subjected to bone marrow examination after return informed consent. Following bone marrow examination the cytochemical stains were performed if there were more than 20% myeloblast or lymphoblast in the bone marrow aspirate smears. Results were interpreted following cytochemical staining and correlated with the morphological findings.

Inclusion criteria- All the patients with abnormal blood morphology in our hospital were subjected to bone marrow examination with returned informed consent.

Exclusion criteria- Old/ known cases of acute leukemia were excluded. Patients with normal blood morphology were excluded.

MPO–gives brownish black granule colour to myeloid series cells as it reacts with the primary azurophilic granules. PAS – reacts with the glycogen of cytoplasm and is specific for ALL. Sudan Black – gives blackish brown discoloration to myeloid series cells. Non specific esterase – reacts with monocytic cells and is positive in AML M4 and AML M5. All the cases of AML were stained with MPO and Sudan black and all cases of ALL with PAS . AML M4 AML M5 with NSE.

REVIEW OF LITERATURE:

The attempt to classify leukemia was initiated by Nikolaus Friedreich in 1857 who categorized leukemia as acute and chronic. In 1868, Neumann used the term "myelogenous " to imply that leukemias arise from bone marrow.[4]Leukemia accounts for 0.15-0.6% of the total admission in general hospitals in India.[5] Males are affected more frequently than females. Male to female ratio in acute leukemia is 3:2. Frequency of leukemia seen in India of AML is 20 -25% and ALL is 15 -25%[5]. The annual incidence rate of AML and ALL is 5.6 and 30.9 per million respectively[6].

Current epidemiological studies predict about 6.4-6.7AML cases per million in USA , while in China it is about 11 AML cases per million and in India 3.5 AML cases per million[7]. ALL is the most common type of acute leukemia diagnosed both in children and adults and is commoner in children[8].

Standard criteria for distinguishing between myeloid and lymphoid acute leukemias were laid down as the first of its kind by French American British (FAB) working group. The WHO classification of leukemia evolved around 1997 with the goal of improving the objectivity and reproductibility which had incorporated cytogenic abnormalities and immunology as the principal designating criteria other than morphology. The major draw back of FAB criteria was failure to incorporate cytogenetics[9].

Morphological examination of the peripheral blood smear and bone marrow aspirate remains the initial mainstay in the diagnosis of acute leukemia[10].

Morphology when coupled with the cytochemistry renders good results in a setting where techniques like immunophenotyping is lacking and cant be thought of especially in rural areas of the country[11].

Belurkar et al[11] studied the correlation between the morphological and cytochemical features of acute leukemia and diagnosed 80% cases of acute leukemia. A total number of 22/33 (66.7%) cases of ALL and 11/12 (91.6%) cases of AML could be assigned correct lineage based on morphology and cytochemical staining. Good results were rendered when morphology was coupled with cytochemistry.

Sachdeva et al[12] carried out a retrospective analysis of all cases of acute leukemia over a period of two years. Out of 469 cases cases of acute leukemia, 193 cases were diagnosed as ALL and 200 cases were diagnosed as AML while 76 cases were diagnosed as acute leukemias cytochemically undifferentiated. Hence only 16% cases of leukemias remained unclassifiable. Thus he concluded that the FAB classification based on morphology and simple cytochemical stains remains effective enough, although cytogenetics and Immunophenotyping can add to diagnostic accuracy in some cases.

Cytochemical stains used in study by Mhaweek et al[13] included sudan black, specific esterase, non specific esterase, periodic acid Schiff and acid phosphatase. Definite diagnoses were made for all 10 of their AML cases, whereas diagnosis were possible in only 79.4 % patient with ALL when morphology and cytochemistry staining was used .

A similar study done by Kheiri et al[14] where they have compared cytochemical and flow cytometric diagnosis in 93 cases of acute leukemia. Their study has shown a lineage agreement of 95.8% cases.

Gupta et al[15]diagnosed 73% cases of AML on the basis of morphological examination . Sudan black was positive in 66% cases of AML and PAS was positive in 22% cases of AML. There were 39% cases constituting of AML M2 which was the highest among all subtypes.

Gupta et all[15]also revealed that PAS stain was positive in 62% cases of ALL. Sudan black was positive in 4.4% cases of ALL. Morphologically 60% of ALL cases were morphologically diagnosed.The mean blast percentage was 85.2% and auer rod was not seen in a single case.

Akram et all[16] studied the value of cytochemical stains in diagnosis of acute leukemia, when cytochemical stains were coupled with morphology 93.3% cases of acute leukemia were diagnosed accurately. A total no of 13/15(86.7%) cases of ALL and 15/15 cases of AML could be diagnosed correctly. The sensitivity for MPO was 86.6%, specificity was 100% and accuracy was 93.33% in cases of acute leukemia AML. The sensitivity of SBB was 100%, specificity was 86.67% and accuracy was 93.3% in cases of acute leukemia. The sensitivity for PAS was 40.3%, specificity was 70% and accuracy was 50% in cases of acute leukemia . The sensitivity for NSE was 50%, specificity was 81.18% and accuracy was 73.3%.

Griffen et all[17]. in a study observed MPO positive in more than 95% cases of AML and PAS positive in 70% cases of ALL.

Ghosh et all[18]in his study found MPO positive in 97% of the cases of AML.

Estey et all[19] in their study of 180 acute leukemia cases found MPO to be very specific marker for the myeloid antigen, the overall positivity of anti MPO in AML was 92% .

However, the advanced, if not sophisticated , approach of diagnostics using cytogenetics and immunophenotyping makes it difficult for developing countries like India to implement WHO classification in routine use. Financial constrain is a major issue in India. Hence some reliable and better alternative is to be suggested outl[19] .

RESULTS:

Out of 250 bone marrow aspirate examinations, 40 cases were diagnosed as acute leukemia on the basis of blast percentage > 20% as per the WHO criteria . The morphological features of the blast cells were examined in the bone marrow aspirate smear on the basis of size of the blast cells, cytoplasmic features, presence or absence of auer rods, granularity in the cytoplasm, nuclear cleavage or lobulations, presence or absence of prominent nucleoli and then the blast were categorized as myeloblast or lymphoblasts.

After the morphological examination the unstained bone marrow aspirate smear was subjected to cytochemical stains that is MPO, PAS, Sudan black and NSE.

18 out 20 cases were positive for MPO giving a bluish to brownish appearance to the granules in the cytoplasm of the blast cells. In two cases of AML, MPO was found to be negative that is a case of AML M6 showing erythroblasts and pronormoblasts and in a case of AML M5.

In 90 % of the cases MPO was found to be positive in AML. Strong positivity for MPO was seen in cases of M1, M2 and M3.

MPO helped out and aided in differentiating between AML and ALL.

PAS stain was positive in a single case of AML M6 and AML M5. PAS positivity was seen in monoblast in AML M5 and the erythroblast in AML M6, however in these cells small blocks of positive material were present against a diffusely positive cytoplasmic background . PAS positivity in AML cases was 10%.

Table 1: Distribution of cases on Bone marrow examination diagnosis.

Distribution of Cases on Bone Marrow Examination	No. of Cases	Percent
Aplastic Anemia	33	13.2
MDS	42	16.8
Normoblastic marrow	13	5.2
Dry tap	2	.8
Megaloblastic Anemia	17	6.8
Hypersplenism	20	8.0
NA	13	5.2
CML AP	57	22.8
CLL	9	3.6
CML CP	3	1.2
AML M1	3	1.2
AML M2	7	2.8
AML M3	5	2.0
AML M4	3	1.2
AML M5	1	.4
AML M6	1	.4
ALL L1	10	4.0
ALL L2	5	2.0
Acute leukemia (Subtyping not done)	5	2.0
Polycthemia Vera	1	.4
Total	250	100.0

Sudan black was found to be positive in 16 cases of AML while negative in four cases of AML. One case of M6, one case of M5, one case of M1 and one case of M4. The reaction product is black and granular. The results are essentially similar to those seen with MPO staining both in normal and leukemic cells.

The only notable difference is in eosinophilic granules, which have a clear core when stained with SBB. The reaction of Sudan black B in blast cells is more defined one with auer rods more prominent. Sudan black positivity in cases of AML was 80%.

PAS stain was positive in 10 cases of ALL while negative in 5 cases of ALL. The reaction product was red, intensity ranging from pink to bright red. Cytoplasmic positivity was showing block like pattern in lymphoblast of ALL. The PAS positivity 66.6%.

Table 2: Distribution of Acute leukemia in the study.

Acute leukemia	Number (%)
AML	20(50%)
ALL	15(37.5%)
Acute leukemia unclassified	05(12.5%)
Total	40(100%)

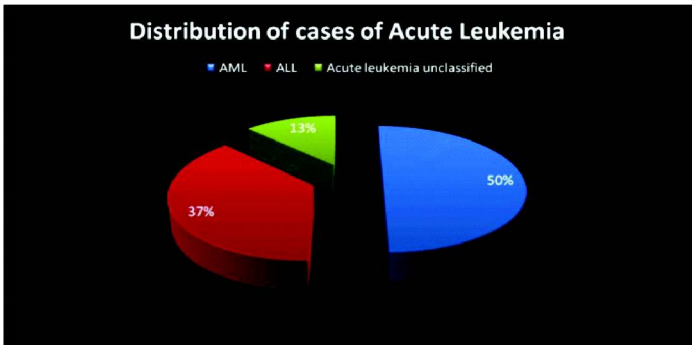


Table 3: MPO staining in cases of AML diagnosed morphologically.

Subtype	AML
M0	0/0
M1	3/3
M2	7/7
M3	5/5
M4	3/3
M5	0/1
M6	0/1
M7	NO CASE

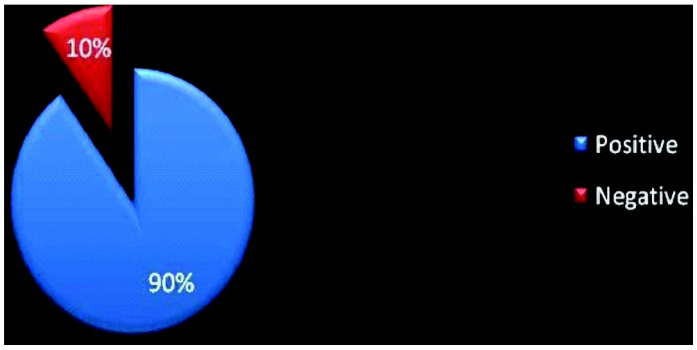


Table 4: SBB Staining in cases of AML.

Subtypes	AML
M0	0/0
M1	2/3
M2	7/7
M3	5/5
M4	3/3
M5	0/1
M6	0/1
M7	NO CASE

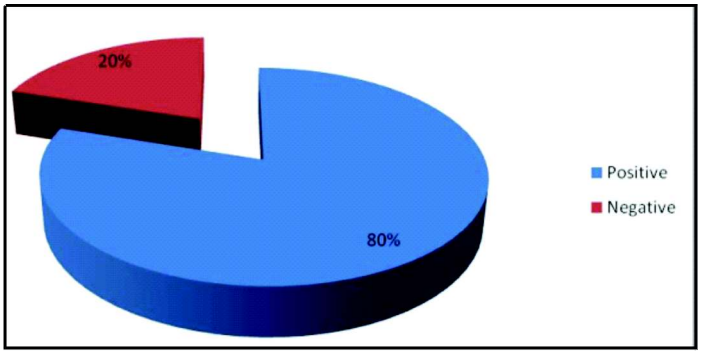


Table 5: PAS staining in cases of ALL.

SUBTYPES	ALL
ALL L1	7/10
ALL L2	3/5

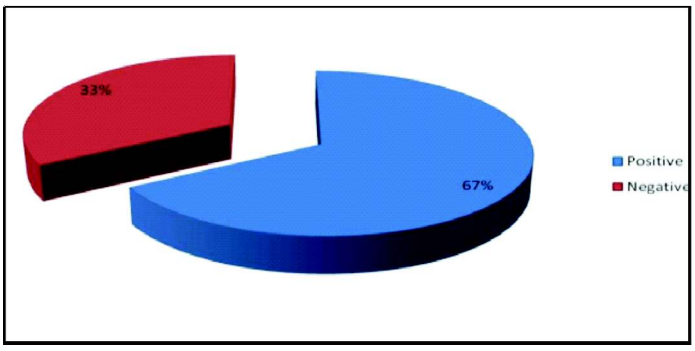


Table 6: Sensitivity, specificity and accuracy of SUDAN in ALL.

		Diagnosis			Sensitivity		80 %
		AML	ALL		Specificity:		100 %
				Total			
SUDAN	Positive	16	0	16	Positive Value	Predictive	100 %
	Negative	4	15	19	Negative Value	Predictive	78.94 %
	Total	20	15	35	False Positive Rate		0 %
					False Negative rate		20 %
					Concordance (Accuracy)		88.57 %

Table 7: Sensitivity, specificity and accuracy of PAS in ALL.

.		Diagnosis			Sensitivity		66.6 %
		ALL	AML	Total	Specificity:		90 %
PAS	Positive	10	2	12	Positive Value	Predictive	83.3 %
	Negative	5	18	23	Negative Value	Predictive	78.2 %
	Total	15	20	35	False Positive Rate		10 %
					False Negative rate		33.3 %
					Concordance (Accuracy)		80 %

MPO, Sudan black and NSE stain were negative in all cases of ALL.

NSE:

NSE was found to be positive for only cases of AML M4 & AML M5. NSE stain was negative for all cases of AML except for AML M4 & AML M5. NSE helped out in distinguishing monocytic and myeloid series.

Trephine biopsy was done in a few cases of AML and ALL and results of trephine biopsy matched with that of morphological features of bone marrow aspirate examination.

Morphological features of blast cells were taken as the golden standard for the assessment of cytochemical stains. In Sudan the control used was unstained smears of known cases of AML giving positivity for SBB. Control- Unstained smears of Known cases of ALL.

The sensitivity of MPO in our study was 90%, specificity was 100% and accuracy was 94.285% in cases of AML. The sensitivity of SBB in our study was 80%, specificity was 100% and accuracy was 88.57% in cases of AML. The sensitivity of PAS in our study was 10%, specificity was 33.3% and accuracy was 20% in cases of AML.

The sensitivity of MPO in our study was 0%, specificity was 10% and accuracy was 5.714% in cases of ALL. The sensitivity of SBB in our study was 0%, specificity was 20% and accuracy was 11.42% in cases of AML. The sensitivity of PAS in our study was 66.66% in cases of ALL. We have used morphological analysis of blast cells in cases of acute leukemia as the golden standard for testing the sensitivity, specificity and accuracy of cytochemical stains. What we have seen morphologically we have confirmed cytochemically.

The p value calculated is significant showing a good correlation between. PAS stain and morphological features of ALL.

The p value calculated is significant showing a good correlation between SBB stain and morphological features of AML .

The p value calculated is significant showing a good correlation between MPO stain and morphological features of AML. The p value calculated is insignificant.

DISCUSSION:

The present study shows the haematological profile of 40 patients suffering from acute leukemia diagnosed on the basis of morphological and cytochemical features. The samples were screened in the peripheral blood film followed by the bone marrow examination. The observation period of the study was from February 2015 to July 2016. The parameters

Table 8: p value of PAS stain.

Stain		Leukemia		Chi Square	p-value
		AML	ALL		
PAS	Negative	18	5		
	Positive	2	10	12.21	0.001
	Total	20	15		

Table 9: p value of SBB.

		Leukemia			
STAIN		AML	ALL	Chi Square	p-value
SUDAN	Negative	4	15		
	Positive	16	0	22.105	0.000
	Total	20	15		

Table 10: p value of MPO.

		Leukeimia			
		AML	ALL	Chi Square	p-value
MPO	Negative	2	15		
	Positive	18	0	27.794	0.000
	Total	20	15		

Table 11: p value of NSE.

		Leukemia			
		AML	ALL	Chi Square	p value
NSE	.00	16	15		
	1.00	4	0	3.387	0.119
	Total	20	15		

taken for the evaluation were peripheral smear, bone marrow examination, cytochemical stains (MPO, PAS, Sudan black &NSE) which was compared with various studies published in the literature.

Total number of cases diagnosed with acute leukaemia was 40. Out of which 20 cases were diagnosed as AML and 15 cases were diagnosed as ALL on the basis of morphological and cytochemical features observed in peripheral blood smear examination and bone marrow examination however 5 cases remained unclassified.

We diagnosed 7 cases of AML M2, 5 cases of AML M3, 3 cases of AML M4 and AML M1 each and single case of AML 5 and AMl M6.

Belurkar et al[11] diagnosed 7 cases of AML M2 which was highest among all the subtypes of AML. Gupta et al[15] diagnosed 39 cases of AML M2 which was highest among all the subtypes of AML.AML M2 was the subtype of AML with highest cases. We in our study also observed highest number of cases of AML M2.

Satheesh et al[5] in his study on acute leukemia has stated that in acute leukemia males are more than females. We in our study have also observed the same.

Table 12: comparison of cases diagnosed on the basis of morphology & cytochemistry .

Comparative Study	% of cases diagnosed on the basis of morphology coupled with cytochemical stains
Belurkar et al. 2013	90%
Saumitra et al. 2009	90.3%
Sachdeva et al. 2006	83.3%
Our Study	87.5%

Table 13: Comparison of MPO, SBB & NSE in various studies.

Study	MPO	Sudan	NSE
Belurkar et al	91.6%	-	-
Mhaweek et al	100%	-	-
Ghost et al	97%	-	-
Griffin et al	95%	-	-
Estey et al	92%	-	-
Gupta et al	-	66%	-
Our Study	90%	80%	20%

In our study the male to female ratio was 1.66:1. Diagnosing acute leukemia has revolusionised over years. With the introduction of WHO classification of myeloid neoplasm techniques like immunophenotyping, FISH, PCR have come in fashion but still the FAB classification has value and is the of prime importance in diagnosing acute leukemia. In the study done by Belurkar et al[11] she concluded that cytochemical analysis of acute leukemia when coupled with morphology rendered the diagnosis in >80% of our acute leukemia. In a set up lacking facilities for immunophenotyping , morphology and cytochemical analysis best serve the purpose in the diagnosis of acute leukemia. A total no of 22/33 (66.7%) ALL cases and 11/12 (91.6%)AML cases were diagnosed. The results of correlation of morphology and cytochemical staining was similar to our study hardly showing any significant changes.

Cytochemical stains used in the study by Mhaweek et al[13] included MPO, PAS& Specific esterase. Definite diagnosis were made for all 10 cases of AML whereas diagnosis was possible in 79.4% cases of ALL. These results were comparable to our study.

Sachdeva et al[12] carried out a retrospective analysis of all cases of acute leukemias over a period of 2 years. Out of which 469 cases of Acute leukemia was diagnosed , 193 cases were diagnosed as ALL and 200 cases as AML. only 16% cases of leukemias remained unclassified. The results of correlation of morphology and cytochemical staining was similar to our study hardly showing any significant variation.

The cytochemical staining aids and helps in analysing the direction of diagnosis in acute leukemias by correlating with the morphology.

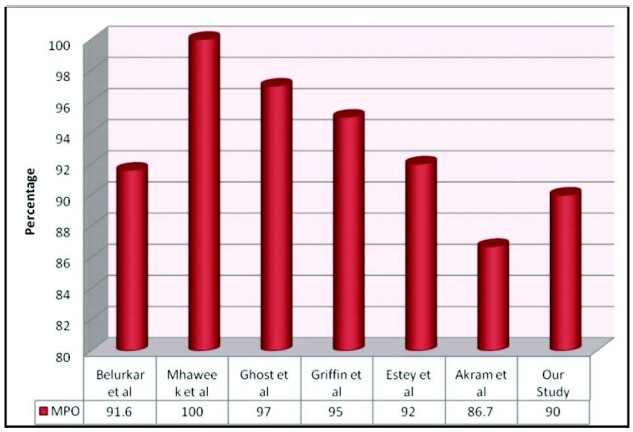
The diagnostic modality of immunophenotyping is very costly and requires a huge capital. In a country like India practically it is very difficult to implement the WHO classification in routine use the morphological and cytochemical analysis is very economical as well as very informative.

MPO showed good results in cases of AML except for a case of AML M5 and AML M6 all other subtypes of AML were positive for AML. MPO is highly specific and sensitive in cases of AML showing good results and positivity.Sudan black B runs parallel with MPO showing good results in cases of AML. MPO has rendered good results in diagnosis acute leukemia in the studies conducted in the past. Our study has shown 90% positivity for AML. SBB is found to give similar results as MPO does in cases of AML. PAS showed 66.6% positivity in cases of ALL in our study .NSE is found to positive in cases of AML M4 & AML M5. Discussion

MPO helps out in differentiating AML from ALL cytochemically. MPO is highly sensitive and specific for AML in our study. We have observed SBB to give more prominent staining in AML than MPO.

The results for PAS stain in ALL was comparable to results of other studies. PAS stain is positive in cases of ALL but not that higly specific as MPO is in case of AML. PAS was positive in 10 cases of ALL but negative in 5 cases. Those five cases were soulely based on morphological examination.PAS plays a important role in diagnosing M5 and M6. The results of PAS stains were compared to other studies.

It is easy to diagnose AML M2 and AML M3 morphologically because of presence of auer rods and increased granularity in the cytoplasm of the blast cells. Moreover AML M2 and AML M3 are strongly positive for MPO and SBB, so cytochemical correlation is good.



Graph: MPO staining results in different studies.

Table 14: Comparison of PAS staining in ALL in different studies.

Study	PAS staining in cases of ALL(%)
Belurkar et al.2013	66.7%
Mhaweek et al .	79.4%
Gupta et al . 2015	62%
Our Study	66.6%

In cases of AML M4 and AML M5 morphologically a clue strikes to the mind is when we see monocytes, monoblast but a cytochemical correlation is must with NSE which aids the diagnosis.

Morphologically it is very difficult to diagnose a undifferentiated blast cell as it mimics features of both a undifferentiated myeloblast and a lymphoblast ALL L1. Morphologically there is no major difference between a undifferentiated myeloblast and a lymphoblast. The common features among both are homogenous population of small to medium sized cells, a very high N:C ratio, basophilic cytoplasm and occasional nuclear cleavage. A major difference between them is presence of prominent nucleoli in undifferentiated myeloblast while in ALL L1 the nucleoli are rarely visible. In some cases ALL L1 shows prominent nuclear cleaving. We have diagnosed three cases of ALL L1 solely on the basis of morphology . In those three cases the size of the blast was small to medium , cytoplasm was basophilic with prominent nuclear cleaving and lobulations which is absent in AML M0 . Cases of ALL L1 with occasional nuclear cleavage create a suspicion for AML M0. In these three cases P

Two cases of ALL L2 have been diagnosed solely on the basis of morphology. The size of blast cell was large with moderate amount of cytoplasm and prominent cleavage, there was no evidence of granularity or auer rods and cytochemically it was negative for all the four stains. Points against AML M1 was prominent nuclear cleavage. At times M1 presents with absence of granularity in cytoplasm but it never shows prominent nuclear cleavage. Occasional nuclear cleavages are seen in AML M0 but it is not prominent.AS, MPO, SBB and NSE were negative.

Two cases of ALL L2 have been diagnosed solely on the basis of morphology. The size of blast cell was large with moderate amount of cytoplasm and prominent cleavage, there was no evidence of granularity or auer rods and cytochemically it was negative for all the four stains. Points against AML M1 was prominent nuclear cleavage. At times M1 presents with absence of granularity in cytoplasm but it never shows prominent nuclear cleavage. Occasional nuclear cleavages are seen in AML M0 but it is not prominent.

In cases of undifferentiated blasts cells cytochemistry fails to give diagnosis as these cells have not yet developed their normal complement of enzymes and metabolic products.

Several studies show that MPO is highly positive for AML. MPO helps in differentiating AML from ALL. It plays a important role in diagnosing AML M1 and differentiating it from ALL L2 but not always. AML M1 with no granularity or little granularity shows a negative MPO staining. Sudan Black B gives a more prominent colouration as compared to MPO PAS is not specific for ALL. It was present in 10 cases of ALL but negative in 5 cases. Those five cases were soulely based on morphological examination. PAS plays a important role in diagnosing M5 and M6.

In the era of Immunophenotyping, PCR & FISH morphological and cytochemical analysis in the cases of acute leukaemia still is of importance especially in developing countries like India where financial burden to the patient is also to be considered especially in rural areas where people have a low socio economic status and cant afford diagnositic procedures like FISH & PCR. Secondly there are a very few Institues in our state and nation having such diagnostic modalities as it requires a huge capital. There are limited number of setups having Immunophenotyping, PCR and FISH in our state.

Morphological and cytochemical analysis in acute leukemias is of a prime importance in a setup lacking Immunophenotyping, PCR and FISH as it is the only mainstay left out at these centres. In huge corporate hospitals, multispeciality hospitals where

immnunophenotyping, PCR and FISH are routinely available even at those places still morphological and cytochemical analysis of acute leukemia is of importance as it is the mainstay in initial diagnosis of acute leukemia giving a direction to further evaluations moreover it helps in assigning correct lineage and reducing the cost to be utilised for immunophenotyping.

In our study most of the patients diagnosed with acute leukemias were of a low socioeconomic status. They could not afford expensive tests like FCA & FISH. In such cases morphological and cytochemical correlation of blast cells is the only best method left out in order to give a accurate diagnosis.

In the present study it is evident that morphological and cytochemical analysis of the blast cells in acute leukemia is very cost efficient, economical handy, easy to do and a reliable method giving good results and is still of prime importance in the era of flow cytometry, FISH and PCR. Morphological and cytochemical analysis in cases of acute leukemia is a blessing in disguise for the people belonging to low socio economic status who can not afford the expensive diagnostic techniques like FCA, PCR & FISH.

Howsoever the latest technology be available morphological and cytochemical features of acute leukemia will remain the main stay in the diagnosis of acute leukemia.

CONCLUSION:

Diagnosing acute leukemia is a step wise process. Firstly we need to distinguish acute leukemia from other hematological malignancies. Secondly we differentiate between AML and ALL and then subtyping is done. Morphological analysis of the blast cells helps out in differentiating between AML and ALL in most of the cases except undifferentiated blasts. Cytochemical stains aid in diagnosing acute leukemias.

Cytochemical stain like MPO gives good results in cases of AML and helps out in differentiating AML from ALL. NSE is very useful in differentiating myeloid series from monocyte series. NSE is very helpful in diagnosing AML M4 and AML M5. Sudan black B is found to give a more prominent staining in cases of AML. PAS is useful in cases of ALL but speficity and accuracy is less.

In the present study it is evident that morphological and cytochemical analysis of the blast cell is a very reliable, cost efficient, economical, handy, easy to use method.

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