CYTOGENETIC STUDY OF CHROMOSOMAL ABERRATIONS ASSOCIATED WITH CHRONIC LEUKEMIA

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ABSTRACT: The most common cause of death is bone marrow failure. Chromosomal analysis is essential for the diagnosis, response to treatment and prognosis of leukemia. The present study was done to know the chromosomal abbreviation in chronic leukemia of patients with in age group of 2 to 60 years. 32 male and 21 female patients have been analyzed for this study. The chosen leukemic patients were those who were reporting for bone marrow examination in the Hematology section of Pathology Department from the coastal District of Orissa. The chromosomal aberrations were taken into account in this present study and arranged in tables, Bar charts and Pie charts for comparison. Identifying the chromosomal changes in leukemic cells will help physicians to use targeted therapy, aimed at the specific genetic defect in the cancer cells responsible for leukaemia. Conventional karyotyping which shows the structural, numerical and compound aberrations have improved the genetic diagnosis of leukaemia. It forms an important platform on which pyramid of modern cytogenetics have been growing for the most accurate diagnosis of leukemia.

INTRODUCTION: Leukaemia’s are cancers arising from unregulated clonal proliferation of hematopoietic stem cells. The failure of feedback control of mutant clonal growth is usually caused by major genetic changes in regulatory gene sites leading to senseless genes and over production of cells, incapable of normal maturation and function. Individual malignant cells mature slowly and incompletely. Their cell cycle time is prolonged and most of these incompetent cells survive longer than normal. As these un-programmed cells reach useless abundance, expanding infiltrates may outgrow their vascular supply and succumb to ischemic necrosis because many tumour cells are still born or die prematurely from frailty and overcrowding. The doubling time of the tumour mass may be vastly protracted beyond that predicted by the cell cycle time.

Leukemia, like other cancers, result from somatic mutations in the genes which facilitate oncogenes or deactivate tumour suppressor genes, and disturb
the regulation of cell death, differentiation or division. These mutations may occur spontaneously or as a result of exposure to radiation or carcinogenic substances. Cohort and case-control studies have linked exposure to petrochemicals, such as benzene and toluene to the development of some forms of leukemia.

MATERIALS AND METHODS:
The material for the present study comprises of, bone marrow of leukemia patients of both sex within the age group of (2 to 60 yrs) out of which 32 male and 21 female patients have been analyzed for this study. The chosen leukemic patients were those who were reporting for bone marrow examination in the Hematology section of Pathology Department from the coastal District of Orissa.

The present study was undertaken in the Dept. of Anatomy, IMS and SUM Hospital, Bhubaneswar in collaboration with Haematology section of Pathology, IMS AND SUM Hospital, BBSR for sample collection.

Criteria for Selection of Patient:
The patients were selected on the basis of clinical diagnosis and pathologically presenting blast cells in the peripheral smear. Patients suffering from pyrexia of unknown origin (PUO) with hepatosplenomegaly sternal tenderness and unexplained anemia were usually taken. Written/informed consent was obtained from the patient for cytogenetic analysis and questions were asked about their family for pedigree chart up to three generations.

Cytogenetic analysis:
- Sample Collection

With the help of a pre--heparinised disposable syringe (0.5-1 ml) of bone marrow was drawn aseptically from posterior superior iliac crest of each leukemic patient. The sample was collected and transferred to a culture bottle containing 5 ml of RPMI 1640, culture media from which 2.5 ml was taken for immediate analysis and the remaining 2.5 ml was added with 1 ml of fetal calf serum for 24 hrs, incubation.

The Cytogenetic study of bone marrow sample was enough to achieve adequate metaphases as it contains plenty of dividing cells. For immediate chromosome analysis 0.01 mg/ml of colchicine was added to 2.5 ml of the sample and kept for 30 minutes followed by hypotonic treatment (0.600mg% KCL) was given for 40 minutes. Fixation was done by methanol and glacial acetic acid in (3:1) proportion.

The remaining 2.5 ml of the bone marrow sample with RPMI 1640 culture media was added with 1 ml of fetal calf serum for 24 hours incubation. The sample was kept for 24 hours in an incubator at 37º C to achieve more dividing cells. Next day 0.01 mg/ml of colchicine was added 1 hrs prior to chromosome analysis to arrest cells at metaphase stage for 20 minutes followed by hypotonic treatment (0.600mg% KCL) was given for 40 minutes. Fixation was done by methanol and glacial acetic acid in (3:1) proportion and harvesting was done as per the protocol.

Slide Preparation:
Preparation of slides by air-drying method was followed after fixation. Clean slides were taken and made grease free by dipping it in chromic acid overnight. Fixed cells suspension (2-3 drops) was dropped on hot and moist slide from a height of 18-24 inches. The slides were kept in vertical position and allowed to spread out for drying. The air-drying method at most times yields a higher number of better metaphase. The dried slides were coded properly by using a marker pencil.

Banding Protocol:
Step 1: The dried and coded slides were kept for 5 to 7 days inside an incubator at 37º C for maturation.

Step 2: The matured slides were treated with Trypsin solution for 20 seconds, which was made in one cuplin jar (trypsin 50 mg /100 ml of distilled water).

Step 3: The trypsin digested slides were rinsed with (NACL 0.9%) twice which were kept in two cuplin jars.
Step 4: The slides were again washed with distilled water and stained with 2 ml of Giemsa stain (4%) which was poured over the slides and kept for 1 minute after which equal amount of distilled water was added for 5 minutes. The stained slides were washed with distilled water thoroughly and allowed for air-drying.

Step 5: The stained and air dried slides were examined under light microscope for screening, which shows alternate light and dark bands on the chromosomes.

Step 6: These slides were screened for 20 well spread metaphases, five of them were photographed and one was karyotyped and studied for chromosomal aberrations.

The enlarged microphotographs were taken and karyotyped manually by considering the length of the chromosome in decreasing order, position of the centromere, presence of satellite bodies and banding pattern.

Examination of slide:
Slides were stained with Giemsa stain and examined under light microscope. The fields selected showed good separation of chromosomes without any overlap or clumping. Five well spread metaphases were selected for photography under 1000x magnification. One good photograph of chromosomes per patient was selected for karyotyping. In cases where it was difficult to distinguish two chromosomes or some artifacts were present in the photograph, rechecking of the same metaphase was done directly through microscopy. Chromosomal abnormalities, which were observed only from karyotypes, were similarly confirmed by rechecking through direct microscopy.

Confirmation of an abnormality was finally made on the basis of both microscopic as well as photographic observation, though main importance was attached to direct microscopic observation because photograph reveals less details of chromosome structure than observed directly in microscope. The chromosomal analysis of all the patients under this observation was done according to the standard protocol. The chromosomes thus obtained were banded, karyotyped and studied in detail for translocations, deletions, inversion, monosomy, trisomy and hyperdiploidy.

RESULTS:
The chromosomal aberrations were taken into account in this present study and arranged in tables, Bar charts and Pie charts for comparison.

Table 1 shows the cases of leukemia of all types combined in the age group of (2-60) years. They are divided into 3 age specific groups i.e. 0-20 years, 21-40 years & 41-60 years. There are 11 cases (20.7%) in the age group of 41 –60 years. There are 4 CML cases seen in this age group. The CLL & CML cases are seen in maximum number in this age group of 41-60 years consisting of 60% of total cases studied.

Table 2 shows 9 CML cases (17%) and 6 CLL cases (11%) have been observed in the present study. They are diagnosed pathologically according to FAB classification. In chronic Lymphoblastic Leukemia (CLL) the number of male patients are almost double than the female. The male-to-female ratio in individual types of leukemia are 1.25:1 for CML & 2:1 for CLL. Hence the male-to-female ratio in all types of leukemia combined is 1.6:1. It is also observed that in all subtypes of leukemia male patients are more prevalent than female.

Table 3 shows two peak incidences of leukemia. The first peak is in the age group of 0-20 years constituting 16 cases due to high age specific incidence of ALL. The second peak is in the age group of 41-60 years constituting 17 cases due to high age specific incidence of AML, CML & CLL. In this table various types of chromosomal aberrations are given in detail. There are three sets of age groups taken into consideration, 0-20 years, 21-40 years & 41-60 years. The structural aberrations are found in equal proportions between the age group of 0-20yrs and 41-60yrs. The numerical aberrations are observed more or less equal in all the age groups, with a slight rise in age group of 41-60yrs. The compound aberrations are observed in the age group of 0-20 years & 41-60 years in equal numbers.
Table 4 shows that the chromosomal aberrations are more in male 29 than female 16, the male-female ratio is 1.8:1. Structural aberrations are more common than numerical. There are four cases of compound aberration present in this table. Numerical aberrations are found more in male than female. The male-to-female ratio is 1.7:1. Compound aberrations are found much higher in male than female. Hence the total chromosomal aberrations are almost double in male than female.

Table 5 shows the cytogenetic observation in the present study. Out of 15 cases, there are 12 abnormal karyotypes (85%) & 3 normal karyotypes (15%). Abnormal karyotypes are seen more in CML cases in comparison to CLL cases. Both normal and abnormal karyotypes are shown in the cytogenetic observation. The normal karyotypes are those having 46 number of chromosome without any alternation in number and structure. The abnormal karyotypes are deviating from the normal chromosome number (46) and classified as structural, numerical & compound sub-groups. The structural aberrations are translocations, deletions and insertions. The numerical aberrations are hyperploidy, hyperdeplody, psudohypoploidy and hypoploidy. Those karyotypes having both structural and numerical abnormalities are classified under compound aberrations.

Table 6 shows 12(90%) cases of chromosomal aberrations in the present study. The structural aberrations are t(9:22) for CML & t(11:14) for CLL. The deletion is also included in structural aberrations in ALL 46XY, 12p-. The numerical aberrations constituted Hypo & Hyperploidy. The example of Hypoploidy are 44XY –5, monosomy 45XX –7 etc. & that of Hyperploidy are Trisomy 47 XY +21, 47 XX +13 &> 50 chromosomes in a metaphase. Hyperdiploidy is the multiples of haploid number i.e. 69 XXX (23X3). The incidence of numerical aberrations is constituted 29% of total chromosomal abnormalities. In the present observation 8(15%) cases are shown to have normal karyotypes having 46 chromosomes. Structural aberrations are seen more in CML cases whereas numerical aberrations are predominant in CLL.

Table 7 shows 9 cases of CML out of which 8 (89%) are Ph positive and 1 (11%) is Ph negative. The number of male and female cases is 5 & 4 and the male female ratio is 1.3:1. The mean and median ages are 37 and 40 years respectively. Out of 8 Ph positive cases, 4(50%) are male and 4(50%) are female. Majority of patients are seen (20-40) years, which constitutes 63% of the total. The incidence of chronic myeloid leukemia is 18% of all leukemia and 23% of myeloid leukemia. The translocations (9:22) are found in 8 patients and one patient shown normal karyotype is described under Ph negative group.

Table 8 shows the total 6 patients of CLL in the present study out of which 4 are male and 2 are female. The male to female ratio is 2:1. The mean and median age is 52.5 years and 56 years respectively. Maximum number of CLL cases is seen in the age group of (55-60) years, which constituted 66.7% of the total cases observed. The male patients suffering of from CLL are almost double than the female patients.

There are 2 numerical, 1 structural and 1 compound aberration found in this study. Two cases have normal karyotypes.

The most frequent chromosomal aberrations are Trisomy-12 in which one extra chromosome was added to the chromosome pair number 12. Also there is one structural and one compound aberration present in addition to Trisomy –12.

DISCUSSION: Chromosomal aberrations associated with leukemia are wide spread and well established. Following the introduction of differential staining techniques an increasing number of non-random chromosomal aberrations has been observed in leukemia. The chromosomal aberrations in leukemia are more than 150 as studied by Rowley et al. (1998) 1.

This present study is a preliminary work to re-establish the findings of the previous workers and to find out new recurring chromosomal aberrations if any. We observed CML & CLL cases were maximum in the age group of 41-60 Yrs (Table 1).
The males are more sufferer than females. The ratio was found to be 1.6:1 in the present study (Table 2).

According to SEER program 1978-1986 for chronic myeloid leukemia, the male-to-female ratio was approximately 1.7:1. The median age at diagnosis was 65 years. It was seen that CML constituted 18.6% of all leukemia in the age group of 45-49 years, and it is most prevalent in persons older than 40 years. Our observation almost corroborates with above workers but there are little variations, which may be due to the small group subjected for analysis & geographical variation 2.

In the present study it was observed that there were two peak incidences of leukemia in the age group of 0-20 years and 41-60 years (Table 3). It was also seen that chromosomal aberrations were more in male than female, the ratio being 1.8:1, (Table 4).

In the observations of Jose. A. Hernandez et al. (1995) the male-to-female ratio for all leukemia combined was approximately 1.7:1 for CLL & CML. There were two distinct age related peaks for all categories of leukemia. The first peak was in children younger than 15 years & progressively the incident declined. The second peak for all leukemia combined was observed in middle 30s and increased progressively. This was due to the progressive increase in age-specific incidence observed in the late 30s or early 40s for CML & CLL 3.

We observed that there were 45(85%) abnormal and 8(15%) normal karyotypes out of 53 cases (Table 5).

In this study the chromosomal aberrations found in leukemia were structural (62%), numerical (29%) and compound (9%) types (Table 6).

Puiet al. (1980) has obtained 89% abnormal karyotypes out of which 56% were structural, 28% were numerical and 5% were compound aberrations 4. Raimondi et al. (1989) had identified 92% chromosomal aberrations of various types in his experiment including (59%) structural and (33%) numerical 5. William et al. (1982) observed that there were 67% abnormal and 33% normal karyotypes in his study on leukemicases 6. Kaushal et al. (2001), had studied 26 patients cytogenetically and it was observed that there were 21 patients (81%) had abnormal and 5 patients (19%) normal karyotypes. The abnormal karyotypes constituted structural, numerical and compound aberrations 7. Konx et al. (2003) had shown abnormal karyotypes in 92% cases including structural, numerical and complex types in his cytogenetic study 8. Jorsova et al. (2003) found 96% abnormal karyotypes of structural, numerical and compound aberrations in cytogenetic as well as molecular basis 9.

Chronic Myeloid Leukemia (CML):

There were 5 male and 4 female patients of CML observed in the present analysis 1:1.25. The male to female ratio was Majority of them were seen in the age group of 21-40 years. Mean and median age was 37 & 40 years respectively.

In the study carried out by S.I. SONATA et al. (1978) there were 57 cases of chronic myelocyticleukemia. The patients were in the age group of 20-68 years. The median age at diagnosis was 41 years, male to female ratio being 1.5:1 10. As per Jorge E Cortis et al (1995) had studied 560 patients with morphologic diagnosis of CML. They were in the age group of 14-71 Yrs. (median age 44 Yrs.). Male to female ratio was 1.3:1 11. C.B JHA et al. (2005) had analysed the bone marrow samples of 19 CML cases. They were in the age group of 15-82 Yrs. There were 14 male and 5 female cases. The male to female ratio was 2.8:1. Majority of the patients (68%) were below the age of 40 years, 4 patients (11.4%) were in between the age of 40-50 years. and 2 (10.5%) patients were above 50 years of age 12.

Our finding is almost corroborating with the above authors.

In our study we had analysed 9 cases of CML out of which 8 cases (89%) had Philadelphia chromosome positive. The remaining 11% were Phnegative (Table 7). Kaushal, S and Sidhu S.S et al. (2001) had studied 14 CML cases, in which 13(92%) patients had abnormal karyotype i.e. Ph+ chromosome 7. Jorge E Cortis et al. (1995) had
studied 560 patients with morphologic diagnosis of CML. Cytogenetic analysis suggested that 508 (91%) cases were Ph positive. The study carried out by S.I. Sonata et al. (1978) in 57 patients, 38 (67%) showed Philadelphia chromosome positive as the only karyotypic anomaly.

Similar karyotypic anomaly with positive Philadelphia chromosome is observed in the present study.

**Chronic Lymphocytic Leukemia (CLL):**

In the present study, four male and two female Chronic Lymphocytic Leukemia (CLL) cases were studied (M/F ratio 2:1). The mean and median age was 52.5 and 56 years respectively. Maximum numbers of CLL (66%) cases were seen in the age group of 55 to 60 years. (Table 8).

Antonio Cuneo & Massimo Balboni (1995) studied 42 cases diagnosed by FAB classification. The median age was 60 years (range 51-78 years). Male to female ratio was 2:1. Alain-Delmer et al. (1995) analysed 32 patients with various B cell chronic lymphoproliferative disorders diagnosed pathologically. They were in the age group of 40-78 Yrs. The median age at diagnosis was 62 Yrs. There were 19 male and 13 female patients. The male to female ratio was 1.5:1. As per the observation of Martin Schorder et al. (1995), the patients in CLL were in the age of 39-81 Yrs. (median 60 Yrs).

In our finding, the median age is 56 years, which is 4-6 years less than the observation of the above authors. These variations may be due to a small group subjected for analysis or racial and geographical variation. In our present study on CLL there were 2 numerical (Trisomy 12), 1 structural t(11:14) and 1 compound 47XX t(11:14), +12 aberrations. The most frequent chromosomal aberrations were trisomy (12) and translocation t(11:14), which consisted 75% of total chromosomal aberrations in CLL. (Table 9).

Antonio Cuneo & Massimo Balboni (1995) studied 42 CLL cases diagnosed by FAB classification. There were 7 cases of translocations t(11:14) six cases of trisomy (12) and 11 cases had other recurring clonal abnormalities. Cytogenetic diagnosis reveals t(11:14) which was typical in CLL. It was concluded that t(11:14) identifies a cytogenetically atypical subset of B cell characterized by frequent cytologic and cytogenetic evolutions.

Alain-Delmer et al. (1995) analysed 32 patients in which 28 patients showed abnormal karyotypes. Translocation t(11:14) (q13 q32) and del (11q13) was found in 18(56%) patients. The other aberrations were chromosome 11q13 either a t(11:14) or a del (11) (q13) without evidence of chromosome 14 involvement and concluded that t(11:14) was the most common translocation in CLL.

As per the observation of Martin Schorder et al. (1995) seventy patients of CLL were analysed cytogenetically. The chromosomal aberrations were trisomy 12 (50%), t(11:14) 30% and others were compound translocations. Only one case had shown unbalanced 9p aberration.

The present finding also corroborates with the reports of the above workers.

P. Kadaret al. (1995) studied 25 Patients with myelodysplastic syndrome (MDS). In the follow up study cytogenetic analysis were repeated after chemotherapy. Six patients did not respond to it whereas 7 patients had good response, 5 of them were in the younger age group of 2-22 years. It was concluded that younger age group had got better treatment response.

In the pedigree analysis it has been observed that two cases are having leukemic trait in the first and second-degree generations constituted 3.8% of all the cases observed in the present study.

The cause of leukemia is not known. Genetic factor is one of the probable causes in its multi factorial etiology, as observed in the pedigree analysis of the present study. All the literatures, works and facts pointed out that chromosomal aberrations are the sine qua non of leukemia. Our work is a small tide in the ocean of cytogenetics so far as leukemia is concerned.
FIG. 1: KARYOTYPE OF BONE MARROW CELL OF FEMALE CML PATIENT SHOWING 46XX t (9:22). THIS ENLARGED KARYOTYPED PHOTOGRAPH IS SHOWING THE DELETION OF LONG ARM OF CHROMOSOME NO 22, WHICH IS RECIPROCALLY TRANSLOCATED TO THE LONG ARM OF CHROMOSOME NO. 9. SUCH TYPE OF CHROMOSOMAL ABERRATION IS KNOWN AS TRANSLOCATION t(9:22)

The enlarged microphotograph of metaphase chromosomes from bone marrow cells

FIG. 2: KARYOTYPE OF BONE MARROW CELL OF MALE CML PATIENT SHOWING 46XY

The above-enlarged karyotyped photograph is having 23 pairs of chromosome without any alternation in number and structure. This is an example of normal study after chemotherapy.

Metaphase Spread:

FIG. 3: KARYOTYPE OF BONE MARROW CELL OF FEMALE CML PATIENT SHOWING 46XX

The above-enlarged karyotyped photograph is having 23 pairs of chromosome without any alternation in number and structure. This is an example of normal study after chemotherapy.
FIG. 4: KARYOTYPE OF BONE MARROW CELL OF FEMALE CML PATIENT SHOWING 46XX, AFTER CHEMOTHERAPY SHOWING GOOD RESPONSE

Metaphase Spread:

This enlarged karyotyped photograph is showing the deletion of long arm of chromosome no 14 which is reciprocally translocated to the long arm of chromosome no 11. Such type of chromosomal aberration is known as translocation 46XY t(11:14).

FIG. 5: KARYOTYPE OF BONE MARROW CELL OF FEMALE CLL PATIENT SHOWING 47XX, +12

Metaphase Spread:

This enlarged karyotyped photograph is showing one extra chromosome in 12th pair. This is known as trisomy 12.

FIG. 6: KARYOTYPE OF BONE MARROW CELL OF FEMALE CLL PATIENT SHOWING 47XX, +12

Metaphase Spread:

KARYOTYPE: 45XY t(8:14), -7
This enlarged karyotyped photograph is showing one extra chromosome in 12th pair. This is known as trisomy 12, the analysis is carried out after chemotherapy showing poor response.
CONCLUSION: Since the study has been a small one, it is not possible to draw a definite conclusion for which a large database analysis is required to establish the accurate fact. Our increasing precision in identifying the chromosomal changes in leukemic cells comes at a most opportune time, because physicians will soon be in a position to use targeted therapy, aimed at the specific genetic defect in the cancer cells responsible for leukaemia. Conventional karyotyping which shows the structural, numerical and compound aberrations have improved the genetic diagnosis of leukaemia. It forms an important platform on which pyramid of modern cyto and molecular genetics have been growing for the most accurate diagnosis of leukaemia. Further, it is felt that an attempt should be made for Fluorescence in situ Hybridization (FISH) to identify the origin of the marker chromosomes, oncogene amplifications, subtle translocations, sub-microscopic deletions and duplications associated with leukemia.

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