Pakistan J. Zool., vol. 40(3), pp. 147-157, 2008.

Clinical and Cytogenetic Analyses in Pakistani Leukemia Patients

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Abstract.- Cytogenetic evaluation at diagnosis of leukemia bears invaluable prognostic significance. Since cytogenetic analysis is neither a routine clinical practice nor such studies have been conducted in Pakistan on a large scale basis, the current study was set out to determining the most common clinical features of leukemia, cytogenetic abnormalities and to correlating these with prognosis, age, sex, socio-economic status and ethnicity. Blood and bone marrow cultures of n=50 male and female leukemia patients were processed for standard G-banding and the karyotypes were analyzed using cytovision system. Clinical analysis revealed the most common presenting features in each type of leukemia including weight loss, fever, bleeding, fatigue, pallor, anemia, bone pains, adenopathies, hepatomegaly and splenomegaly. Cytogenetic analysis demonstrated in 69.5% ALL patients a hyperdiploid condition. Structural abnormalities observed were: t(9;22)(q34;q11) and t(1; 19)(q23; p13.3). One patient had trisomy 21 and another had 11q23 abnormality associated with poor prognosis. Hypodiploidy was seen in one patient with a loss of chromosome 20. In AML patients good prognosis was seen in majority with translocations t(8; 21)(q22; q22) and t(15; 17) (q22; q12), while a less percentage showed poor prognosis associated with 11q23 and inv (3)(q21; q26). Majority of CML patients were Philadelphia positive with t(9; 22)(q34.1; q11.2) which questions its rarity in our region; only one was Philadelphia negative having poor prognosis. The single CLL patient had trisomy 12 associated with poor prognosis. Herein we conclude that a combination of clinical and cytogenetic analyses is an invaluable tool in risk stratification of leukemia and its treatment modification.

Key words: Leukemia, cytogenetics, tumor cancer, karyotype, ALL, AML, CML, CLL.

INTRODUCTION

The incidence of leukemia across the world is 1 per 100,000 per year and contributes to 25% of childhood cancers (Cartwright, 1992). Most common types of leukemia are; acute lymphoblastic leukemia (ALL), acute myelogenous leukemia (AML), chronic myelogenous leukemia (CML) and chronic lymphocytic leukemia (CLL). ALL and AML are further characterized into subtypes based on French American British (FAB) classification (Bennett *et al*., 1985). The WHO classification is now based on cytogenetic and clinical features of the AML and has been devised in an attempt to define entities that are biologically homogenous and show prognostic and therapeutic relevance (Strupp *et al*., 2003).

Clinical symptoms and pathologies are although of good prognostic value but the analysis of cytogenetic abnormalities is a very useful prognostic tool for the diagnosis and treatment of leukemia for two main reasons; to confirm the diagnosis of a particular form of leukemia and, to select the best treatment for each kind of patient (Heim and Mitelman, 1992). Also, classical cytogenetic analysis through karyotyping is an invaluable tool for monitoring engraftment and disease status following hematopoietic stem cell transplantation (HSCT) (Otero *et al*., 2007).

A large number of numeric (hyper- or hypodiploid conditions etc) and structural cytogenetic abnormalities: translocations, inversions, deletions, duplications, insertions and amplifications have been discovered in different types of leukemia. In the ALL, translocations t(9; 22) (q34; q11), t(8; 14), t(8, 2), t(8; 22) and t(1; 19) are of good prognostic value at diagnosis (Aricò *et al*., 2000; Ballerini *et al*., 2002; Ernst *et al*., 2002). In the AML, t(8; 21), inv(16) and t(15; 17) are

0030-9923/2008/0003-0147 $ 8.00/0

associated with good prognosis. Normal cytogenetics portends average-risk AML, while patients characterized by deletions of the long arms or monosomies of chromosomes 5 or 7; by translocations or inversions of chromosome 3, t(6, 9), t(9; 22); or by abnormalities of chromosome 11q23 have particularly poor prognosis with chemotherapy (Mrózek *et al*., 2001). In the CML, 90-95% patients have the Philadelphia chromosome whereby chromosome 22 appears to be shortened and is due to the standard t(9; 22) (q34.1; q11.2) reciprocal translocation (Saglio *et al*., 2002). In the CLL, the common anomaly associated is trisomy 12 (Juliusson *et al*., 1991), but its significance as regards prognosis in B-CLL is controversial. Besides this, structural abnormalities of chromosome 13 and 14 are also known (Que *et al*., 1993).

Despite the fact that the prognostic and diagnostic values of cytogenetic abnormalities of leukemia have been established worldwide, data from Pakistan are scanty and hardly if any studies have actually been done from this point of view. Moreover, cytogenetic analysis is not routinely performed at presentation in local hospitals. The present study was conducted with a view to investigating cytogenetic abnormalities in each type of leukemia, correlating them with prognosis and clinical findings in a sample from Pakistani leukemia patients.

MATERIALS AND METHODS

Sample collection

The present clinical and cytogenetic study was carried out on randomly selected patients (n=50: 35 males and 15 females; age range 1-75 yrs) of acute and chronic lymphoid and myeloid leukemia. Inclusion criteria were: patients of either ALL, AML, CML or CLL diagnosed on morphological examination of bone marrow biopsy specimen or by the use of special stains and immunophenotyping wherever required. Exclusion criteria were: patients receiving chemotherapy and those who were in complete remission after receiving one cycle of chemotherapy. There was no history of prior exposure to any sort of radiations and no personal or family history of any hematological or malignant disease in all selected cases.

All patients were selected from out patient department and oncology wards of main hospital and children hospital of Pakistan Institute of Medical Sciences (PIMS) Islamabad, Pakistan. The study period was January through December 2005. Clinical examination included detailed history, presenting complaints, history of drug use, family history and socio-economic status. Physical examination of each patient included pallor, fever, purpura, petechiae, ecchymosis, gingival hypertrophy, lymphadenopathy, hepatomegaly, splenomegaly and the examination of the central nervous system. All details were recorded on carefully designed proformas.

The study was conducted according to the guidelines provided by the ethics committee Pakistan Medical Research Council, Islamabad on medical research on human subjects. The study procedures were explained to all patients and written informed consent was obtained from each patient prior commencement of the study.

Preparation of blood and bone marrow cultures

Cytogenetic studies were conducted on the peripheral blood (3 ml by venipucture collected in sterile vacutainers containing sodium heparin as an anticoagulant) and bone marrow samples (1.5 ml aspirate in RPMI1640 culture medium with L-glutamine, (GIBCO, USA) and 20% fetal calf serum (Sigma, USA) of the same patients who were chosen for clinical studies. Cell density was checked on a blood CP machine (Sysmex, Japan). Standard cytogenetic techniques were used throughout. Cultures of bone marrow and blood were set up for 24, 48 and 72 hours. Phytohemagglutinin (PHA) was added as a mitogen to the blood cultures. 0.04mg/ml colcemid (GIBCO, USA) was added before harvesting; for which, tubes were centrifuged and 0.075M KCl was added to the resuspended pellet, mixed well and incubated at 37°C for 15 minutes; centrifuged again and freshly prepared cold fixative (3:1 methanol and glacial acetic acid) was added to the pellet and centrifugations were performed again 2-3 times. Cell suspensions thus obtained were used to prepare chromosomal spreads.

G-banding and screening

For G-banding, aged slides of metaphase chromosomes were placed for a few seconds in HBSS (Gibco), trypsinized for 60 seconds (6.25mg/ml trypsin in disodium phosphate buffer pH 7.0) and stained with 2% Giemsa stain. Each slide was screened for well banded metaphase spreads and the positions for at least 20 good spreads were recorded using an England finder. Well spread chromosomes were screened and observed with an x100 objective under oil.

Karyotyping

Karyotyping was done according to ISCN guidelines for Human cytogenetic nomenclature using cytovision system for image analysis.

RESULTS

Clinical analysis

Of 50 leukemia patients 23 (46%) were diagnosed with ALL, 15 (30%) with AML, 11 (22%) with CML and 1 (2%) with CLL.

ALL

Of 23 patients, 21 (14 males, 7 females) belonged to the pediatric age group (91%) between 1-15 years with an average age of eleven years at presentation, 2 (females) were adults of ages 23 and 35 years. Patients younger than five years of age belonged mostly to the Punjab province while patients older than this were mostly from the N.W.F.P and Northern areas. Eighteen patients belonged to lower socioeconomic status, three to lower middle class and 100 to higher middle class. One patient was a diagnosed case of Down's syndrome. Patients were presented with complaints of bone pains involving lower sternum and joints. All were anemic and lymphadenopathic with variable degree of involvement of cervical, axillary and inguinal lymph nodes. Mild to moderate hepatomegaly was a common observation. Splenomegaly was moderate; petechiae, purpura and ecchymosis were observed either alone or in combination. None of the patients showed signs of raised intracranial pressure or testicular involvement (Table I).

AML

Of fifteen (5 females, 10 males) patients, nine (60%) belonged to pediatric age group (age range 7-18 yrs) with an average age of fifteen years at presentation. The remaining six patients were adults with an average age of 39 years at presentation. Most (n=10) belonged to the Punjab province, three were from the Northern areas and two from Swat district. Ten patients belonged to lower socioeconomic status while five were from lower middle class. All patients complained weakness, fever, fatigue, a variable degree of weight loss and mucosal bleeding. All were anemic, had gingival hypertrophy and a variable degree of lymphadenopathy involving cervical, axillary and inguinal lymph nodes. Some patients had mild to moderate hepatomegaly and mild splenomegaly (Table I). Morphological examination of the bone

Table I.- Clinical findings in leukemia patients.

Clinical features	Frequency (%)
ALL (n=23)	
Weakness, weight loss, pallor, hepatomegaly, lymphoadenopathy	100% (n=23)
Fever, splenomegaly	78% (n=18)
Bleeding	91% (n=21)
Fatigue	43% (n=10)
Bone pain	22% (n=22)
Signs of raised intracranial pressure	0% (n=0)
Testicluar Involvement	0% (n=0)
AML (n=15)	
Weakness, pallor, fatigue	100% (n=15)
Fever	60% (n=9)
Bleeding	53% (n=8)
Hepatomegaly	40% (n=6)
Gingival hypertrophy, splenomegaly	27% (n=4)
Lymphadenopathy	20% (n=3)
CML (n=11)	
Fatigue, splenomegaly	100% (n=11)
Hepatomegaly	82% (n=9)
Weight loss	73% (n=8)
Ecchymosis	18% (n=2)
CLL (n=1)	
Weakness, fatigue, weight loos, pallor, lymphadenopathy, hepatomegaly, splenomegaly	100% (n=1)

marrow and special staining procedures revealed following sub types according to the FAB classification of AML. Four patients were diagnosed with M1 (26.6%), five with M2 (33.3%), three with M3 (20%), two with M5 (13.3%) and one with M7 (6.6%).

CML

Of eleven patients (9 males, 2 females), the average age at presentation was 18 years with an age range of 14-55 years. All CML patients were from the Punjab province. Nine belonged to lower middle class, while two had a lower socioeconomic status. All complained fatigue and a variable degree of weight loss. Mild to moderate hepatomegaly, moderate to massive splenomegaly and ecchymosis were the outcomes in these patients (Table I).

CLL

CLL was diagnosed in a single male patient who was 75 years old. The patient was from the Punjab province and belonged to the upper middle class. The patient was presented with complaints of weakness, fatigue and weight loss. He was anemic with painless cervical lymphadenopathy, moderate hepatomegaly and massive splenomegaly (Table I).

Cytogenetic analysis

Cytogenetic analysis was performed on all 50 patients who were selected for clinical analysis while 3 males and 3 females healthy individuals were taken as control. Karyotype was 46 XY and 46 XX in normal males and females, respectively. Of several figures, only the normal male and female, karyotypes and one figure each for the numerical and structural karyotypic abnormalities in AML are presented here as a representation of the cytogenetic data (Figs. 1, 2).

ALL

Of 23 ALL patients, for numerical abnormalities, hyperdiploid karyotype (Fig. 1) was observed in 16 (69.5%) patients, all belonging to the pediatric age group. Out of these sixteen, 10 (62.5%) patients had 47-50 chromosomes and five (31.2 %) patients showed >50 chromosomes while one patient had trisomy 21 (6.25%). Hypodiploid karyotype was observed in one (4.3%) pediatric patient with the loss of chromosome 20. Patients having normal Karyotype with standard risk were five (21.7%) pediatric and one (4.3%) adult patients. Among structural abnormalities; t(9; 22) (q34; q11) was observed in one (4.3%) adult patient and t(1; 19) (q23; p13.3) (Fig. 2) was observed in one (4.3%) pediatric patient (Table II).

Table II.- Cytogenetic findings in leukemia patients

	Cytogenetic abnormality	**Frequency (%)**
ALL (n=23)		
Numeric abnormalities	Hyperdiploid	69.5% (n=16)
	47-50 chromososmes	62.5% (n =10)
	>50 chromosomes	31.2% (n = 5)
	hypodiploid	4.3% (n = 1)
	Trisomic	6.25% (n = 1)
Structural abnormalities	Pseudodiploid t(9;22) (q34;q11)	4.3% (n = 1)
	t(8;14), t(8;2) and t(8;22)	0% (n = 0)
	t(1;19)(q23;p13.3)	4.3% (n = 1)
AML (n=15) (FAB classification)		
M1	11q23	6.6% (n = 1)
	(diploid)	20% (n = 3)
M2	t(8;21)(q22;q22)	26.6% (n = 4)
	Diploid	6.6% (n = 1)
M3	t(15;17)(q22;q12)	6.6% (n = 1)
	Diploid	13.3% (n = 2)
M5	11q23	6.6% (n = 1)
	Diploid	6.6% (n = 1)
M7	inv(3)(q23;q26)	6.6% (n = 1)
CML (n=11)		
Ph positive	t(9;22)(q34;q11.2)	91% (n = 10)
Ph pegative	diploid	9% (n = 1)
CLL (n=1)		
	Trisomy 12	--- (n = 1)

AML

Of fifteen AML patients, four had M1 morphology, of which three (20%) had normal karyotype and one (6.6%) had 11q23 abnormality. Of five patients with M2 morphology, four (26.6%) had t(8; 21) (q22; q22) abnormality, whereas karyotype was normal in one (6.6%) patient. Among three patients with M3 morphology, karyotype was

normal in two (13.3%) patients and one (6.6%) had t(15; 17) (q22; q12). Out of two patients with M5 morphology; one (6.6%) had 11q23 abnormality and

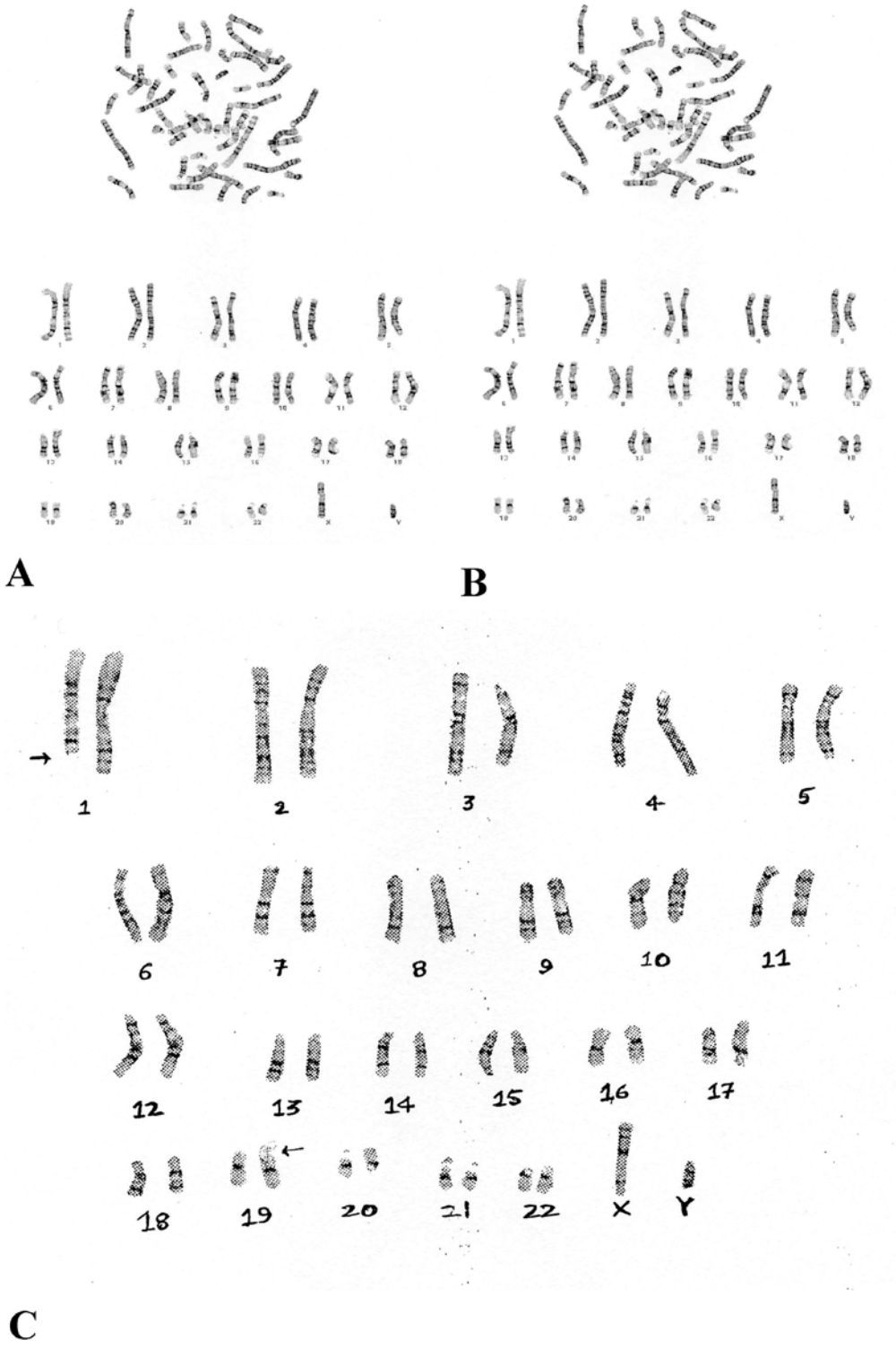

Fig. 1. Human G-banded chromosomes showing normal karyotypes for male (A) and female (B), (C) arrows show structural abnormality, translocation t(1;19) (q22;p13.3) karyotype in ALL.

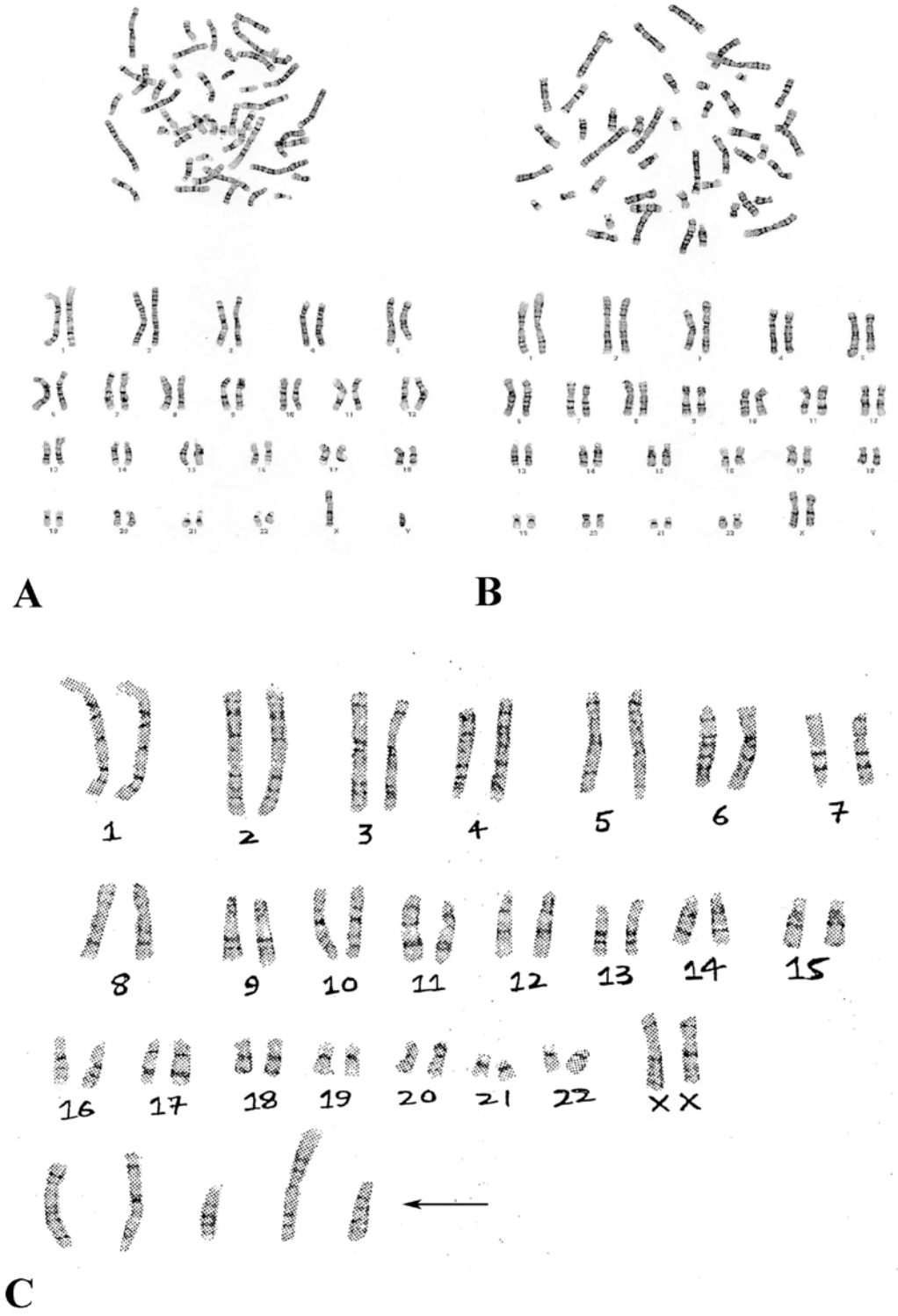

Fig. 2. Human G-banded chromosomes showing normal karyotypes for male (A) and female (B), (C) arrow shows numerical abnormality, hyperdiploid karyotype in ALL.

another one (6.6%) had a normal karyotype. One (6.6%) patient with M7 morphology had inv (3) (q21; q26) (Table II).

CML

Of eleven patients, ten (91.0%) patients had a Ph positive karyotype with t(9; 22) (q34.1; q11.2) chromosomal abnormality whereas one (9.0%) patient was Ph negative with diploid karyotype (Table II).

CLL

The single patient of CLL had trisomy 12 (Table II).

DISCUSSION

Cytogenetic analysis in ALL

Pediatric age group was found to be the most prevalent in ALL with an average age of eleven years at presentation and a male predominance; an observation similar to Ries *et al*. (1991). Both patients in the adult age group were females, in contrast to the cases reported in literature where adult patients of ALL were reported to have male predominance and much older age group (Brincker, 1982). In the present study ALL was found more prevalent in individuals from N.W.F.P and Northern areas as compared to the Punjab province, but unfortunately no data is available to compare. Most patients with ALL belonged to lower socioeconomic status which is in contrast to earlier reports where children with a better socioeconomic status were shown to be afflicted with the disease (McWhirter, 1982). Nutritional status and socioeconomic factors may influence the prognosis of ALL in children. Socioeconomic status is usually revealed at presentation while taking the history of the patient. Some hospitals in Pakistan do cater for the needs of poor and needy in terms of consultation and provision of medicine but it is not a very widespread practice which obviously demands attention. Black children have been reported to have poorer survival rates compared with whites. Asian survival rate is as good for Whites, but Hispanics have poorer survival rates than Whites (Bhatia, 2004). We found in our ALL patients, a poor prognosis for girls than boys, an observation already reported by Pui *et al*. (2004). It appears therefore that the socioeconomic factor does play important role in the good or bad prognosis of the disease. Since Pakistan is a land of people with widely different ethnic or racial backgrounds, there is a need to determine the influence of socioeconomic factor on the prognosis of ALL by conducting large scale studies in children from different ethnic, racial or social backgrounds.

Children with ALL who present with CNS disease at diagnosis are at higher risk for treatment failure (Burger *et al*., 2003); however, CNS disorders were not encountered presently in any of our ALL patients. The most common initial symptoms observed were anemia, neutropenia, and thrombocytopenia which were manifested by fatigue, weakness, fever, weight loss, and bleeding. In the present study, however, 100% of the patients were presented with lymphadenopathy and hepatomegaly while splenomegaly was found in almost 80% of the patients. Involvement of the lungs, heart, eyes and gastrointestinal tract was less frequent. Contrary to the present report, studies from other countries indicate 80% lymphadenopathy; 70% to 75% hepatomegaly and/or Splenomegaly (Scheinberg *et al*., 2001). Skin involvement is seldom seen and is almost always associated with the pre-B-cell phenotype (Henderson, 1990). Presently also, skin abnormalities were not seen in any of the ALL patients. Hyperdiploidy (>50 chromosomes per cell or DNA index >1.16) occurs in 20-25% of cases (Raimondi *et al*., 1992); but in contrast, during the present study, hyperdiploidy was observed in 70% cases. A similar proportion of hyperdiploid karyotype has been reported in Iranian children (Farkhondeh *et al*., 2001). This and the present study therefore by obvious reasons show the important finding of a greater prevalence of hyperdiploidy in Asian region of the world. However, hyperdiploidy is generally associated with favorable prognosis. It is known that trisomies 4, 10, and 17 are usually associated with a potentially favorable prognosis (Hann *et al*., 200; Pui *et al*., 2004).

Presently, Philadelphia positive ALL was seen in one of the two adults with ALL. It is known to be found in 15 to 30 % of adults with ALL and is associated with poor prognosis (Arico *et al*., 2000). Thus clinical and cytogenetic analyses have a crucial role in diagnosis, risk stratification, treatment and prognosis of ALL (Carroll *et al*., 2003). Studies of Chinese population with ALL in 124 pediatric patients revealed that 68 cases (60%) had clonal abonormalities. Numerical imbalances encountered were hyperdiploidy (36 cases; 32%), hypodiploidy (14 cases; 12.5%) and pseudodilpoidy (18 cases; 16%). Chromosomal translocations found in 13 patients were: 4; 11, 9; 22 and 1; 19 (Chai *et al*., 2007).

Of 1425 ALL pediatric (0-14.9 yrs old) patients from Sweden and Denmark, it was discovered that in 2-7 years old, 80% non-Down B-cell prescursor ALL cases had a high-hyperdiploid clone (51-61 chromosomes) or a translocation t(12; 21) (p13; q22). B-cell precursor ALL cases had 11q23/MLL-aberrations, translocation t(12; 21) (p13; q22) and high-hyperdiploidy. The study demonstrated that "the incidence peaks of the childhood acute leukemias reflect specific cytogenetic aberrations" (Forestier and Schmiegelow, 2006). In a study in Taiwanese children with ALL, in 78 patients of under 18 years of age, 20.5% had normal diploidy; 35.9% had pseudodiploidy; 7.7% with hyperdiploidy (47-50 chromosomes); 24.4% with hyperdiploidy (>50 chromosomes) and 99.4% had hypodiploidy. Most frequent structural abnormality detected was t(9; 22) (Chang *et al*., 2006).

Clinical and cytogenetic analyses in AML

A male preponderance in AML patients was observed which is in accordance with the previous studies (Kosary *et al*., 1995). Although, we accept that the sample size was small, in contrast to the observations in ALL cases, AML appears to be more prevalent in the Punjab province than the N.W.F.P and Northern area. Unfortunately, no possible explanation for this observation has as yet been found and no data is available for comparison. The most common clinical features presented were the same as already known and included: anemia, neutropenia and thrombocytopenia.

Recent studies (Vandana *et al*., 2003) describe a more favorable outcome for children with t(8;21) which was observed in four out of five patients with M2 morphology. AML with t(15;17) was observed presently in a single patient and was invariably associated with APL, a distinct subtype of AML that is treated differently than other types of AML because of its marked sensitivity to the differentiating effects of all-*trans* retinoic acid (Micallef *et al*., 2001). Parallel to ours report, t(15;17) incidence has been reported in three of five patients with M3 morphology in an Iranian study (Farkhondeh *et al*., 2001). Anwar *et al*. (2006) have recently demonstrated in Pakistani AML patients that majority of the patients belonged to good (23%) or standard (65.4%) risk groups; whereas 11.6% patients belonged to poor risk group. Cytogenetic abnormalities with good prognosis were: t(8; 21), t(15; 17) and inv (16) while patients carrying poor prognosis had trisomy 8 and dup (3)(q21; q26). It is noteworthy that we also observed similar abnormalities and further confirm theirs observations.

Translocations of chromosomal band 11q23 involving the MLL gene, are associated with monocytic differentiation (FAB M4 and M5) and in general have poor prognosis. Chromosomal abnormalities associated with poorer prognosis in adults with AML include those involving chromosome 7 (monosomy 7 and del (7q), chromosome 5 (monosomy 5 and del (5q) and the long arm of chromosome 3; inv (3) (q21; q26) or t(3;3)(q21; q26) (Mistry *et al*., 2003).

Data from ethnic Omani population (63 patients) showed that in case of AML, M2 subtype was most common and, 18% children and 44% adults had chromosomal abnormalities. Balanced translocations, t(8; 21) and t(15; 17) were observed in 11% and 10% respectively, Inv (16) was seen in 3% patients. Trisomy 8 was the most frequent numerical anomaly, found in 11% patients while monosomy 7 was seen in 7% patients (Udayakumar *et al*., 2007). As M2 appears to be the most common in our patients also, it is tempting to conclude that Pakistani population is this aspect falls closer to the other populations in the Asiatic region. In 66 Turkish AML patients studied by Sahin *et al*. (2007), chromosomal abnormalities were detected

cytogenetically in 25.7% but molecular cytogenetic analysis detected abnormalities in 31.8% patients, whereas 57.6% patients had normal karyotype. Most prevalent chromosomal abnormalities were t(15; 17), inv 16 or t(8; 21) which showed complete remission during clinical follow up.

Clinical and cytogenetic analyses in CML

All CML patients were in the chronic phase of the disease. Chronic myeloid leukemia is characteristically a tri-phasic disease in which the chronic phase usually lasts for three to six years followed by transformation to accelerated phase and blast crisis. Common features at presentation include fatigue, weight loss, and signs or symptoms of splenomegaly (Silver, 2003; Alvarez *et al*., 2007). The disease accounts for 7% to 15% of all adult leukemias, and occurs slightly more often in men as was currently observed. Although median age at presentation is 40 to 60 years, 10% of patients are under the age of 20 years. The cause is unknown and most cases are sporadic (Cortes *et al*., 1998). Even though our sample of leukemia patients was small but it does indicate relatively greater (22%) incidence of CML in our population. Cytogenetic analysis revealed that 91.0% patients were Ph positive, a detection, which appears to be the major finding of the present study since Ph positive abnormality in CML is a rarity in this part of world (Geary, 2000; Goldman and Junia, 2003). It is known that the Ph chromosome, with the *bcr-abl* oncogene detectable at the molecular level, is present at diagnosis in 95% of patients (Luthra *et al*., 2004). In the present case, a single patient of CML had a Ph negative karyotype. Bcr/abl negative CML is a distinct clinical entity and is associated with very poor prognosis. Cytogenetic analysis is therefore particularly important in CML because the prognosis of Ph positive and Ph negative CML are entirely different (Onida *et al*., 1997).

Clinical and cytogenetic analysis in CLL

A single male aged 75 years old was found afflicted with CLL. However, this is not surprising because CLL constitutes 20% of all leukemias in the Western hemisphere, whereas in Asiatic countries, it accounts for only 2.5% (Keating, 1994). Moreover, the incidence is also age dependent and the male to female ratio is 2:1. The single patient encountered during the present study was presented with complaints of weakness, weight loss and lymphadenopathy with hepatosplenomegaly. It is well known that most CLL patients are diagnosed accidentally on routine examination and are asymptomatic. Only a small number of patients present with particular complaints such as the patient under observation. The patient had trisomy 12 that indicates disease progression and is associated with poor prognosis (Dierlamm *et al*., 1997).

Presently, no single causative agent or risk factor could be identified during the course of the disease. This may have been due to lack of awareness among general public regarding leukemia. It was however observed during the current study that some particular types of leukemia were more common in a particular geographical region. Further such studies are required to be conducted on a large scale to examine identifiable risk factors.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. Khalid Hassan, Professor of Hematology, PIMS, Islamabad for providing access to leukemia patients and valuable suggestions during the study.

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(Received 10 October 2007, revised 14 March 2008)