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Segregation Analysis of p53, c-myc and DNA Ploidy Using Flow Cytometry Among Egyptian Families with Childhood Leukemia

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The present study aimed to check for segregation of some oncogenic markers (p53, c-myc) and DNA ploidy pattern in Egyptian families of children with acute lymphoblastic leukemia (ALL) to determine whether there is an actual risk for cancer among these families. This case-controlled study included 20 Egyptian children with ALL, their median age 6.5 years (interquartiles 2.1-12.5) with males/females 16/4. They were enrolled at presentation to Haematology-Oncology Unit of Mansoura University Children's Hospital, Egypt. The study included also their first-degree relatives (20 fathers, 20 mothers, 44 healthy sibs) and control group; 20 healthy subjects with median age 6.9 years (interquartiles 3.2-13.1) and sure negative family history of cancer. Blood sampling was done for all persons followed by cell isolation for Flow Cytometric analysis (FCM) of DNA ploidy, apoptosis and other cell cycle parameters. p53 and c-myc protein expression were also assessed by FCM using monoclonal-antibody staining technique. p53 and c-myc showed significantly high values in cases compared to controls and relatives ($p < 0.001$) with no difference between parents and sibs. Almost the same differences were noted for cell cycle parameters; G1 and G2M channels, but aneuploid cell% and DNA index showed no statistical differences between relatives and control. Likewise S-phase% lacked differences between relatives and control but significantly lowered in cases compared to relatives and controls. Mating between parents with high p53 and c-myc resulted in high levels in most affected offsprings and more than half of normal sibs. Aneuploid positivity among cases and sibs was segregating independently from parents. Segregation of p53, c-myc expression and DNA cycle parameters among families of ALL children illustrates a semi-dominant inheritance indicating the presence of an underlying genetic and/or environmental factor(s) supporting the theory of familial cancer risk.

Key words: Familial risk, childhood leukemia, flow cytometry, p53, c-myc, ploidy pattern

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INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common childhood malignancy representing almost one third of all pediatric cancers. Annual incidence of ALL is about 30 cases per million populations, with peak incidence at age of 2-5 years (Dalmasso *et al.*, 2005; Wilkinson *et al.*, 2005). It is a malignant disorder resulting from clonal proliferation of lymphoid cells which exhibit markers in earliest stages. Like most malignant tumors it shows an alteration of the genetic material inside cells. In the last five years, there has been a tremendous increase in understanding the molecular genetics of several childhood cancers. Three major classes of cancer genes exist; firstly oncogenes that encode regulatory proteins for cellular growth forcing cells into uncontrolled cell division; c-myc is an oncogene that produces protein phosphates activating CDK₂ and CDK₄ stimulating cell cycle, also c-myc interferes with p27 function (a CDK inhibitor) leading to cell entry into S-phase and finally triggering apoptosis (Rudolph *et al.*, 1996; Medema *et al.*, 1997). Secondly, Tumour Suppressor Genes (TSG) that encode proteins responsible for down-regulation of cell growth leading to important brakes in cell cycle allowing for either DNA repair or apoptosis if DNA damage is so severe. Retinoblastoma and p53 genes are examples of TSG; p53 plays an important role in regulation of cell proliferation so that loss of its function leads to cell transformation *in vitro* and development of neoplasm *in vivo* (Chang *et al.*, 1993). p53 also stimulates DNA repair by interacting with the proliferating cell nuclear antigen and excision repair enzyme (Smith *et al.*, 1996) however if DNA damage is too great, p53 triggers apoptosis pathway through Bax which is a member of Bcl2 family (Bennett *et al.*, 1998). Thirdly is DNA repair genes; a group of genes encoding enzymes that regulate DNA replication and repair by scanning newly-formed DNA molecules, removing erroneously incorporated base pairs and reconstructing corrected DNA molecule (Liu *et al.*, 1996). Aneuploidy is generally associated with malignancy and usually signifies a worse prognosis however it is associated with improved survival in rhabdomyosarcoma, neuroblastoma, multiple myeloma and childhood ALL (Gollin and Janecka, 1994).

Measurement of nuclear DNA content and cell cycle parameters could be done by flow cytometry (FCM) using fluorescent dyes such as propidium iodide, that intercalate into DNA helical structure. The fluorescent signal is directly proportional to the amount of nuclear DNA and thus could identify gross gains or losses in DNA (Dugue, 1993). The aim of this study is to check for segregation of some oncogenic markers (p53, c-myc) and

DNA ploidy pattern using FCM in the first degree relatives (parents and sibs) of ALL children attempting to detect any risk factors for malignancy among these family members.

MATERIALS AND METHODS

This study included 20 children having ALL and their 1st degree relatives; 20 fathers, 20 mothers and 44 siblings (21 brothers and 23 sisters). Patients' age ranged between 2-12 years [median (Interquartiles, IQ); 6.5 years (2.1-12.5)] with males/females ratio 16/4; they were enrolled before induction chemotherapy at Haematology-Oncology Unit of Mansoura University Children's Hospital which is the main referral center of the middle Delta Region of Egypt. Twenty healthy unrelated persons with definitive negative family history of cancer were taken from patients attending outpatient clinics for irrelevant causes and used as a control group; they were of matched age and sex with median age 6.9 years (IQ; 3.2-13.1). After obtaining informed consent, all studied subjects underwent thorough history taking and clinical examination followed by FCM analysis for DNA content, ploidy pattern, cell cycle parameters and apoptosis in addition to p53 and c-myc expression.

Preparation of cell suspension for FCM: Peripheral blood samples (3-5 mL⁻¹) were withdrawn under complete aseptic precautions in EDTA containing tubes followed by isolation of mononuclear cells using ficoll hypaque layering. Cell suspension was centrifuged; pellet suspended in Phosphate Buffered Saline (PBS) and cell count was adjusted to 10⁶ cells mL⁻¹.

FCM for DNA content: Cells were fixed using cold absolute ethanol for at least 12 h. Staining was done using propidium iodide in combination with ribonuclease for RNA digestion using DNA cycle test kit (Dako, Denmark) (Vindelov, 1997). FCM analysis was done using FACS caliber Flow cytometer (Becton Dickinson, Sunnyvale, CA, USA) with built-in computer programs; Cellquest and Modfit. The average number of evaluated nuclei per specimen was 20,000; with the rate of nuclei scanning is 120 sec⁻¹. Cell cycle parameters included G1 (gap1 peak channel), S phase% (percentage of cells in synthesis phase), G2/M (gap2, mitosis peak channel), G2/M aneuploidy (G2/M peak channel for aneuploid cell population), DI (DNA index; ratio of G1 aneuploid/G1 diploid) and apoptosis% (percentage of apoptotic cells measured in the sub-G1 peak). Figure 1 showed FCM demonstration of increased apoptotic cells.

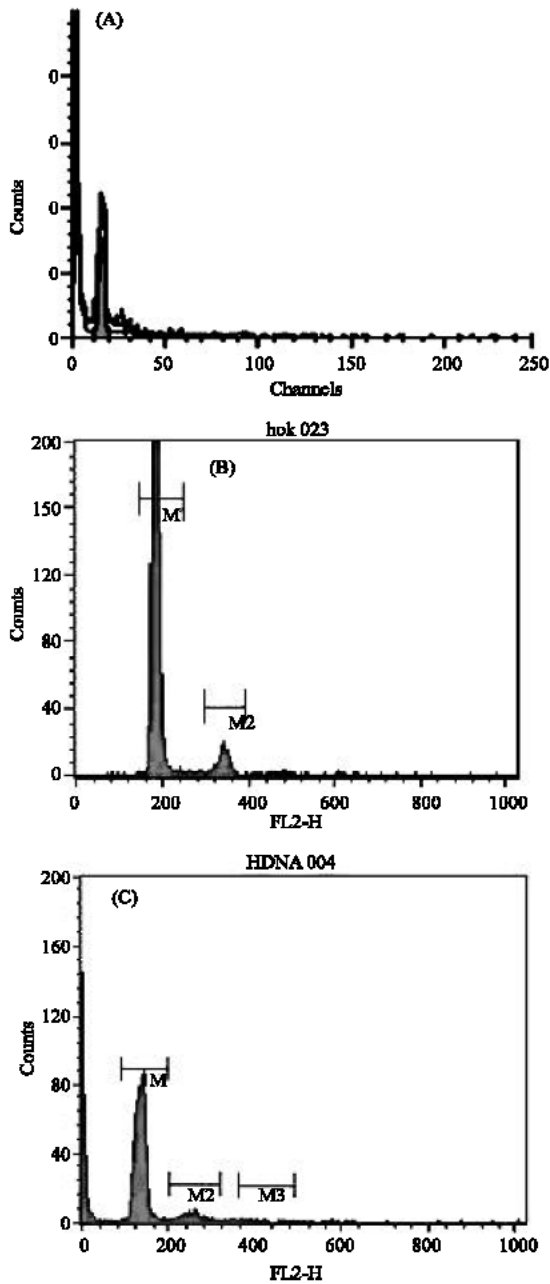


Fig. 1: Flow cytometric analysis of DNA ploidy showing increased apoptotic cells (A), Normal diploid pattern (B) and Aneuploid pattern (C)

p53 Staining method: As the antibody is directed against an intracytoplasmic antigen, permeabilisation of the membrane is necessary before incubation with antibody. To 100 μ L of cell suspension, 2 mL 1% paraformaldehyde in PBS were added dropwise under constant vortexing, incubated for 5 min at room temperature; then 1 mL 100% cold methanol (20°C) added under vortexing. Incubate for

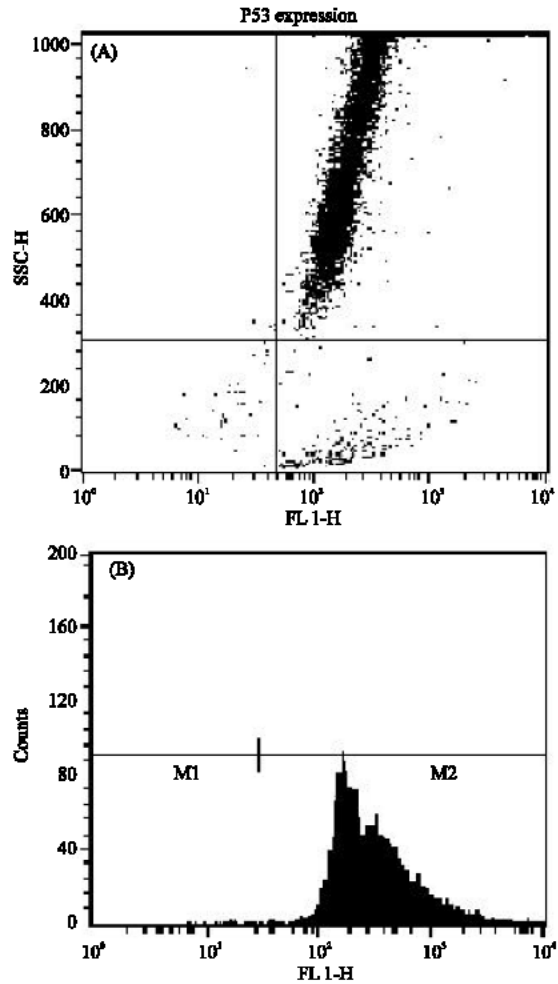


Fig. 2: Flow cytometric analysis of p53 expression on mononuclear cells showing dot plot diagram (A) and histogram (B) of positively stained cells in relation to negative ones

10 min at 8°C; then centrifuge 5 min at 1000 RPM and finally aspirate supernatant; washing cells with PBS and centrifugation repeated 2-3 times. Cells were fixed in paraformaldehyde (0.5% in PBS), mixed with up to 10 μ L of antihuman (p53) (p53-monoclonal antibody labeled with FITC, Dako, Denmark) and incubated in dark at 4°C for 30 min. Washing, centrifugation and aspiration of supernatant were repeated and finally cells were resuspended in appropriate volume of PBS to be ready for FCM analysis (Pudie *et al.*, 1991). The detected p53 is the mutant type (half life equals 30 min up to 6 h) but normal p53 protein has a shorter half life (6-20 min) (Finlay *et al.*, 1988).

C-myc staining method: Aliquot 1 mL of cell suspension to a polystyrene tube and pellet cells by centrifugation at

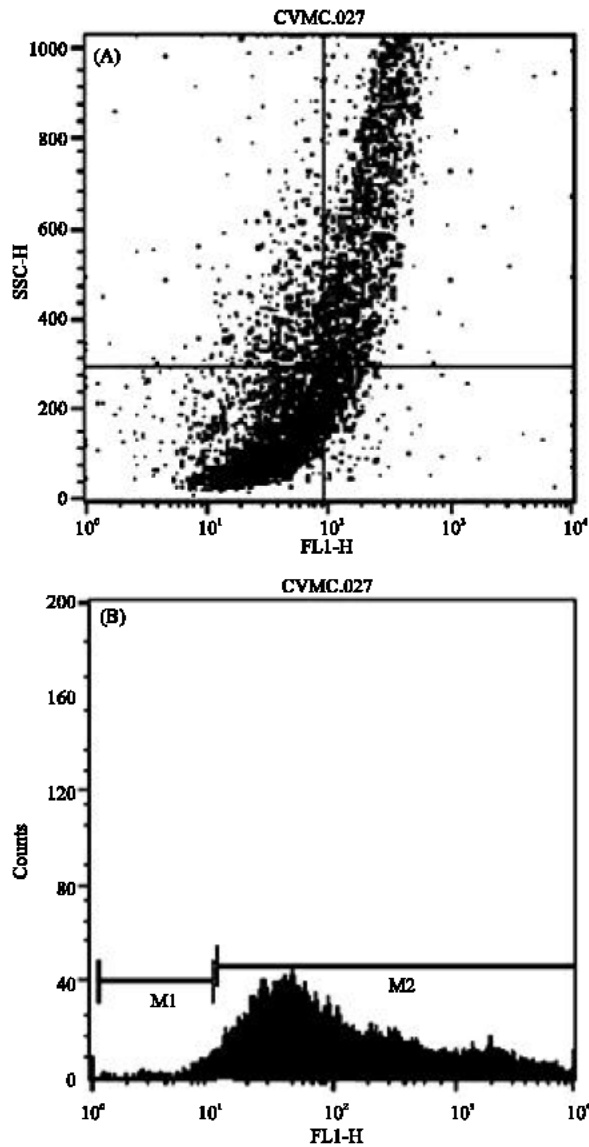


Fig. 3: Flow cytometric analysis of c-myc expression on mononuclear cells showing dot plot diagram (A) and histogram (B) of positively stained cells in relation to negative ones

1000 rpm for 5 min. Pellet was resuspended in 100 μ L buffer mix (0.1% NaN₃ and 0.5% BSA in PBS) to which 3 μ g c-myc monoclonal antibody (Santa Cruz-Biotechnology, USA) was added. A negative control tube was used to which 5 μ g of the appropriate negative control serum was added. Gentle vortexing of samples, incubation on ice for 30 min, washing with 1 mL buffer mix, vortexing again, centrifugation and aspiration of the buffer were done. The whole process was repeated then pellet resuspended in 100 μ L buffer mix. Add 3 μ g

fluorochrome-conjugated secondary antibody [Mouse immunoglobulins FITC Rabbit F (ab), Dako Company, Denmark]; vortexing and incubation of tubes on ice for 1 h being covered away from light. Washing with 1 mL buffer mix, vortexing, centrifugation and aspiration of buffer was repeated 2-3 times before final resuspension of cell pellets in appropriate amount of PBS to be ready for FCM analysis. Figure 2 and 3 showed FCM dot plot diagram (A) and histogram (B) of p53 and c-myc expression respectively on positively stained mononuclear cells in relation to negative ones.

Statistical analysis: Data were processed and analyzed using the statistical package for social science (SPSS, 1999). Exploration for data normality was done by evaluating the differences between medians and means, degree of skewness and kurtosis as well as stem-and-leaf plots; violation of normality was found in most parameters especially tumour markers but G1 and G2M channel parameters showed preserved normality. We applied non-parametric tests for analysis of differences between each two groups; Mann-Whitney-U-Wilcoxon test expressing median (50th percentile) and interquartiles, IQ (25th, 75th percentiles) for central tendency and dispersion of all variables including the parametric ones. Error bars were done plotting the median values and 5th and 95th percentiles as a confidence interval (CI) for c-myc (A), p53 (B) and aneuploidy positive cells © in all studied groups (Fig. 4).

RESULTS

Regarding p53 and c-myc positive cell populations; they were significantly higher in cases compared to other groups (controls, parents and sibs), with no difference found between parents and sibs but both groups had significantly higher values more than controls. This means that relatives attained intermediate values between cases and controls (Fig. 4 A and B).

Levels of p53 and c-myc were considered normal if lying within 5th and 95th percentiles of the studied controls, low if less than 5th percentile and high if more than 95th percentile of control (Levine, 1997; Agrawal *et al.*, 1994). Testing parental mating on this basis (Table 1) showed that mating between parents with high p53 values (High \times High) resulted in 94% of affected offsprings and 89% of healthy sibs having similar high values. Similarly mating between high c-myc parents resulted in 100% of affected sons and 77% of their healthy sibs with high values. Interestingly also, mating between

Table 1: Segregation of p53, c-myc and DNA ploidy pattern among different parental matings and their offspring (cases and normal sibs)

Type of parental matting	Cases (20)					Sibs (44)			
	No.	Total No.	High No. (%)	Normal No. (%)	Low No. (%)	Total No.	High No. (%)	Normal No. (%)	Low No. (%)
P 53									
High×High	(16)	16	15 (94)	1 (6)	0 (0)	37	33 (89)	3 (8)	1 (3)
High×Normal	(4)	4	4 (100)	0 (0)	0 (0)	7	3 (43)	4 (57)	0 (0)
C-myc									
High×High	(16)	16	16 (100)	0 (0)	0 (0)	35	27 (77)	8 (23)	0 (0)
High×Normal	(4)	4	4 (100)	0	0 (0)	9	8 (89)	1 (11)	0 (0)
Aneuploidy			Positive No. (%)	Negative No. (%)			Positive No. (%)	Negative No. (%)	
Positive×Positive	(0)	0	0 (0)	0 (0)	0	0 (0)	0 (0)	0 (0)	
Positive×Negative	(4)	4	3 (75)	1 (25)	7	0 (0)	7 (100)		
Negative×Negative	(16)	16	11 (68.75)	5 (31.25)	37	3 (8.11)	34 (91.89)		

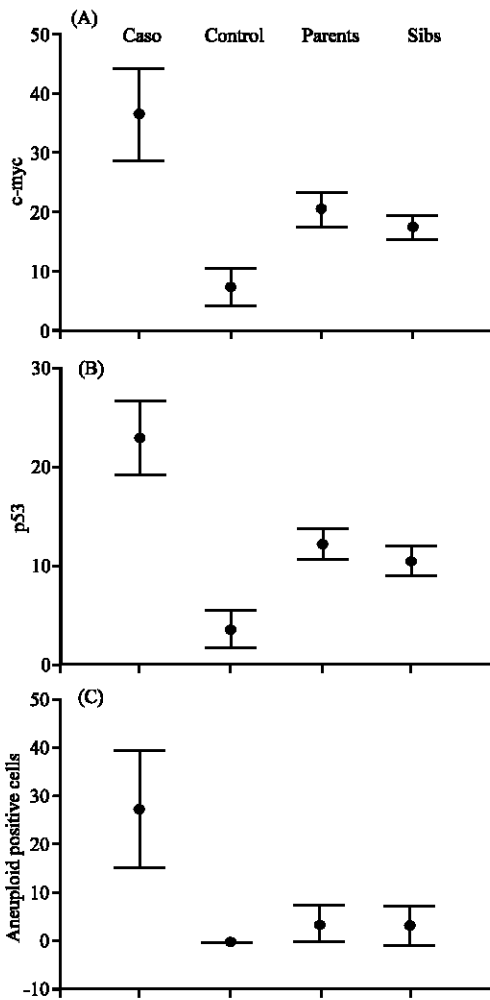


Fig. 4: Standard error bars presenting median values and 5th and 95th percentiles of c-myc (A), p53 (B) and aneuploid positive cells (C) in cases, controls and relatives (parents and sibs) showing higher levels in cases compared to other groups. Note the intermediate values of relatives between those of cases and controls.

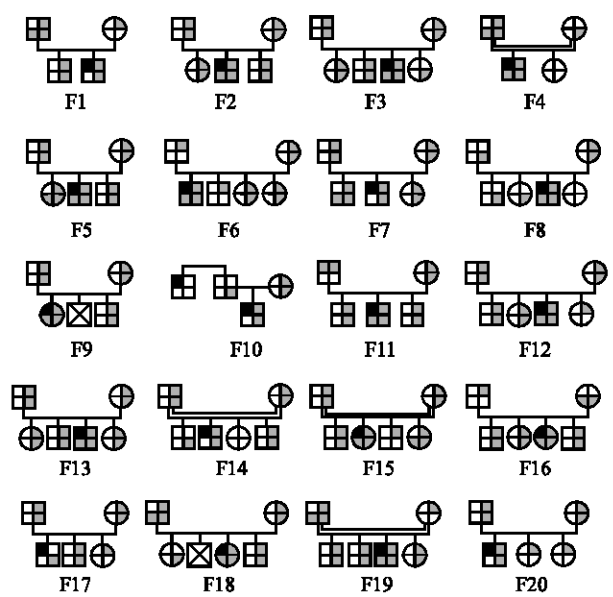


Fig. 5: Segregation of p53, c-myc and DNA aneuploidy among studied families

Relative → □ ← Normal p 53
 -ve aneuploidy → □ ← Normal c-myc
 Cases → ■ ← High p 53
 +ve aneuploidy → □ ← High c-myc

Note: The same legend is applied on female members of the pedigrees (circles)

normal level parents with high level ones resulted in 100% affected sons and about 50% of their healthy sibs having high values meaning that a semi-dominant effect is controlling segregation of these proteins. On the other hand aneuploid positivity was found to be segregating among cases and normal sibs independently from parents (Table 1 and Pedigrees in Fig. 5).

The same was noted on analysis of cell cycle parameters; G1 and G2M channel peaks showed significant higher values among cases followed by those of parents and sibs above those of the controls.

Table 2: Flow cytometric parameters of cell cycle, p53 and c-myc among cases and their 1st degree relatives compared to controls and their significance using Mann-Whitney Wilcoxon U-test expressing Median and Interquartiles; IQ (25th-75th)

Flow parameters	Cases N= 20 Median (IQ)	Parents N= 40 Median (IQ)	Sib N= 44 Median (IQ)	Control N= 20 Median (IQ)	P					
					Cases Vs Controls	Cases Vs Parents	Cases Vs Sibs	Parents Vs Controls	Sibs Vs Controls	Parents Vs Sibs
G1	44.3	40.25	41.3	37.0	< 0.001	0.02	0.04	0.002	< 0.001	0.28
Channel	(40.1-46.5)	(36.9-44.1)	(39.8-44.1)	(36.6-37.8)	**	*	*	*	**	**
G2M	86.4	79.5	81.0	74.0	< 0.001	0.01	0.01	0.008	< 0.001	0.44
Channel	(78.9-92.2)	(73.6-86.2)	(77.0-84.0)	(73.2-75.6)	**	*	*	*	**	**
S-phase	0.0	1.82	2.91	1.78	< 0.001	0.006	0.003	0.62	0.74	0.53
Cell%	(0.0-0.9)	(0.0-6.9)	(0.0-9.4)	(0.5-3.1)	**	*	*			
DNA	1.54	1.15	1.13	1.10	< 0.001	< 0.001	< 0.001	0.34	0.54	0.87
Index	(0.7-2.2)	(0.98-1.91)	(0.95-1.71)	(0.91-1.21)	**	**	**			
Apoptotic	0.74	0.33	0.54	2.31	0.55	0.06	0.21	0.01	0.03	0.19
Cell%	(0.17-5.62)	(0.0-1.75)	(0.16-2.23)	(1.14-6.13)	*	*				
Aneuploid	116.3	0.0	0.0	0.0	< 0.001	< 0.001	< 0.001	0.14	0.23	0.61
Cell%	(74.4-125.4)				**	**	**			
P53 +ve	24.9	12.2	11.2	2.15	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.16
Cell%	(16.1-29.7)	(9.2-15.38)	(7.0-14.4)	(1.7-3.2)	**	**	**	**	**	**
c-myc +ve	34.4	18.3	17.5	3.1	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.14
Cell%	(27.9-44.9)	(13.9-26.6)	(13.8-21.2)	(2.4-12.3)	**	**	**	**	**	**

* Significant if < 0.01 ** Highly significant if < 0.001

Aneuploid cell% and DNA index have also shown higher significant values among cases compared to other groups (controls, parents and sibs) with no significant difference between parents and sibs although both were higher than controls (Table 2 and Fig. 4C). S-phase cell% showed higher levels among parents and sibs than cases and controls while apoptotic cell% did not show any statistical difference between cases and other groups.

DISCUSSION

Familial clustering of chronic lymphatic leukemia and other lympho-proliferative disorders was reported in a number of case reports and multi-generational pedigrees illustrating a vertical transmission consistent with autosomal dominant inheritance (Horwitz *et al.*, 1996; Yuille *et al.*, 1998; Domingo-Domenech *et al.*, 2005). Leukemia may be associated with other familial neoplastic diseases in the context of p53 gene mutations e.g., Li-Fraumeni syndrome and neurofibromatosis type 1 (Felix *et al.*, 1992; Stiller *et al.*, 1994). Initial epidemiologic studies of childhood ALL did not show any increased risk of malignancy among offspring of ALL survivors (Hasle and Olsen, 1997), however some reports showed a risk for childhood ALL among relatives of solid tumors patients (Smulevich *et al.*, 1999; Perrillat *et al.*, 2001). In Utah an association between leukemia and colorectal cancer (CRC) was found suggesting an undetermined familial factor (Hemminki and Li, 2004). Positive family history of haematopoietic malignancies among relatives was found to be associated with a slight increased risk of childhood ALL; but risk of ALL was not increased in children with a family history of cancers other than those of haematopoietic system; thus suggesting a modest

familial contribution to childhood ALL (Infante-Rivard and Guiguet, 2004).

FCM could rapidly analyze multiple parameters of individual cells including size, cytoplasmic complexity, DNA or RNA content and wide range of membrane-bound intracellular proteins (Brown and Wittwer, 2000). DNA histogram plot is widely used to estimate DNA ploidy of tumour cells and cycle parameters particularly S-phase fraction (Jourdan *et al.*, 2002; Takes *et al.*, 2002). Analysis of DNA ploidy may aid in interpretation of borderline lesions e.g., reactive versus neoplastic. In our study, 60% of ALL patients were hyperdiploid (DNA index more than 1.0) with a significant difference between ALL patients and controls regarding G1, G2M, G2 aneuploidy, diploidy and aneuploidy percentage; similar to the reported difference between malignant and benign cells (Takes *et al.*, 2002; Grierson *et al.*, 1995). Lower S-phase fraction was shown in our cases compared to parents, sibs and controls despite of the high G1 peak similar to some reports that showed low coefficient of variation (CV %) due to high proportion of cells in G1; in literature S-phase fraction varied from low to high in different neoplasms due to the heterogeneity of cell populations (Stary *et al.*, 1992; Schmid *et al.*, 1999; Lindh *et al.*, 1984).

In the current study, the significant high values in G1 and G2M channels found among 1st degree relatives compared to the healthy controls could be explained by existence of a heritable genetic factor or exposure to a common environment that predispose relatives to cancer; a point which must warrant further investigations. Recently, retrospective analysis of pure familial haematological malignancies; familial leukemia, lymphoma and myeloma occurring in apparently healthy persons with no antecedent predisposing syndrome

revealed a prominent anticipation supporting the concept of germline transmission of susceptibility gene(s); however linkage to HLA was noticed in some families and deletions in chromosome 7 was described with familial AML (Segel and Lichtman, 2004). Interestingly in our study the intermediate values in G1 and G2M channels noticed among healthy relatives between values of cases and controls might be a beneficial demarcation between benign and malignant diagnosis (Takes *et al.*, 2002; Lorenzato *et al.*, 2002). Pedigree analysis for our families (Fig. 5) showed aneuploidy in 10% (4 out of 40) of the studied parents, in 70% (14 out of 20) of their leukaemic children and 6.8% (3 out of 44) of the healthy siblings with no statistical difference between parents and siblings.

p53 gene mutation was found in up to 50% of human cancers and its protein could be detected by FCM with standardization of the estimated values to the 95% CI of control (Levine, 1997; Agrawal *et al.*, 1994). In this study, we found a significantly high p53 protein in ALL patients compared to controls; in agreement with other reports (Koeffler *et al.*, 1986; Sahu and Das, 2002); moreover, p53 was significantly higher in ALL patients compared to 1st degree relatives and in the later compared to control denoting that relatives attained intermediate values between patients and controls; similar to G1 and G2M channels. On basis of pedigree analysis, among the 16 families (80%) in which both parents had high p53 levels (HXH), 94% of their affected children and 89% of normal sibs had high p53 while the remainder was normal. Also among the 4 families (20%) with high and normal p53 parents (HXN), 100% of cases and 43% of normal siblings had high p53; thus suggesting a dominant transmission. Up to our knowledge no previous studies evaluated p53 protein segregation among families of leukaemic children; however p53 mutation had been identified in familial chronic lymphocytic leukemia (Agrawal *et al.*, 1994). This study also showed significant difference in p53 protein expression among patients with DNA aneuploid cells compared to those with diploid DNA (data not shown); similar to results in primary meningioma that showed higher p53 in aneuploid compared to diploid tumors (Perry *et al.*, 1998).

C-myc is a nuclear protein whose expression is tightly controlled by its extremely short half life; it is thought to play an important role in a number of cellular processes including proliferation and apoptosis; its oncogenic properties are due to its constitutive expression. Recent efforts have been directed towards understanding the function of c-myc protein in cancer biology for therapeutic insights (Dang *et al.*, 1999). In this study, c-myc showed significantly higher values in ALL patients

compared to relatives and controls and higher in the former compared to the latter in a fashion similar to p53. Moreover analysis of our pedigrees showed that the 16 families with high c-myc couples as well as the 4 families with high and normal couples transmit the high c-myc protein to all affected children and more than half of the healthy sibs; thus illustrating also a dominant inheritance. Translocation of chromosome 8 on chromosome 2, 14 or 22 had been described in B-cell leukemia juxtapose c-myc proto-oncogene to immunoglobulin gene leading to an increased c-myc protein expression and promotion of lymphoid malignancies (Marcu *et al.*, 1992; Amati *et al.*, 1993). However, the role of c-myc protein for leukaemic cell proliferation was denied despite the relation of its gene to leukaemogenesis (Tidd *et al.*, 2001).

Finally we can conclude that p53 and c-myc segregation among the studied Egyptian families with cases of ALL suggested an autosomal dominant inheritance with variable penetrance based upon their presence in more than one generation and in both sexes. With the definitely known role of p53 and c-myc in leukaemogenesis together with the high incidence of aneuploidy among haematological malignancies, these parameters could be used collectively as high risk factors among the 1st degree relatives of leukaemic children. An observational follow up for these relatives over an extended period must be allowed to check for additional risks aiding in leukaemogenesis.

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