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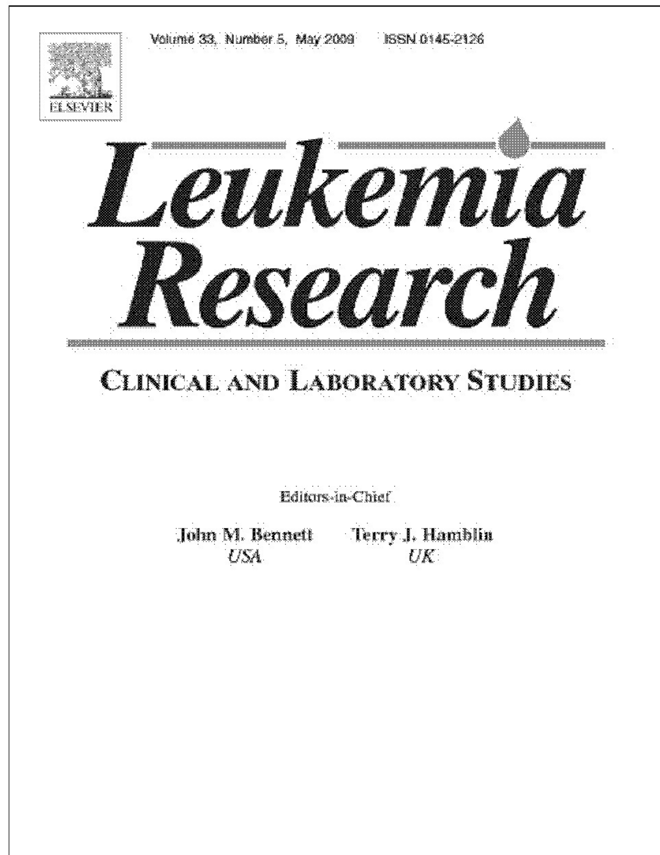

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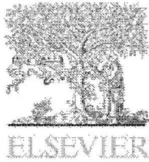

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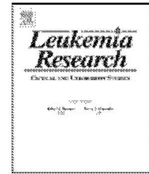

Immunophenotyping and immunoglobulin heavy chain gene rearrangement analysis in cerebrospinal fluid of pediatric patients with acute lymphoblastic leukemia

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ABSTRACT

The study aimed to assess the diagnostic accuracy of Flow cytometry (FCM) immunophenotyping and IgH gene rearrangements (IGHRs) by real-time PCR in comparison with classic cytology for diagnosing CNS infiltration in pediatric ALL. We concluded that the diagnostic value of FCM and IGHR are two to three times more than that of cytology. Therefore, immunophenotyping by FCM is recommended for routine diagnosis of CSF infiltration. Furthermore, IGHR analysis by real-time PCR appears to be a useful addition in evaluation of CNS infiltration.

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1. Introduction

Meningeal involvement is a frequent complication of hematological malignancies with an incidence of up to 25% in certain leukaemias and lymphomas [1]. The diagnosis of this involvement has great prognostic and therapeutic implications both in symptomatic and asymptomatic patients at high risk of such involvement [2]. A diagnostic gold standard is not available, and morphologic examination of cerebrospinal fluid (CSF) fails to demonstrate malignant cells in up to 45% of patients in whom meningeal involvement is thought to be present [3]. The major diagnostic problem in evaluating CSF involvement is distinguishing neoplastic infiltrates from inflammatory or infectious diseases [4].

Flow cytometry (FCM) immunophenotyping is a valuable tool in the diagnosis and staging of lymphoproliferative disorders involving the lymph nodes, blood and bone marrow. While flow cytometric analysis is a standard procedure in the evaluation of blood and bone marrow cells, it is not generally applied to CSF samples in all clinical laboratories [5].

In addition, analysis of heavy chain gene (IgH) rearrangements by real-time polymerase chain reaction (PCR) is a powerful diagnostic tool for hematologists and oncologists. The detection of malignant cells by this technique has become the state of art for diagnosis, monitoring response to treatment and detection of minimal residual disease in leukemia and lymphoma [6]. The development of high technologies allows the application of real-time PCR assays in large prospective treatment studies for monitoring tumor cells in circulation as well as in bone marrow. Based on quantitative data the kinetics of disappearance and reappearance of tumor cells can be followed up in "real-time". This allows developing new strategies to treat patients with an inadequate response to standard chemotherapy or at molecular relapse before symptoms or signs of clinical relapse occur [7]. However, this modality has not been widely applied to CSF specimens [8].

In this study, we aimed to assess the diagnostic accuracy of flow cytometric immunophenotyping and IgH gene rearrangements (IGHRs) analysis by real-time PCR in comparison with classic cytology for diagnosing central nervous system (CNS) infiltration in acute lymphoblastic leukemia (ALL). In addition, we aimed at verifying the CNS status especially at first presentation, which is essential for risk stratification and proper treatment.

2. Materials and methods

Forty-five CSF specimens from pediatric patients with ALL were examined by FCM for immunophenotyping. In 12 patients, CSF analysis was performed because

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Table 1a
Sequences of the consensus primers for IgH used in the first round PCR.

Sequences of the consensus primers for IgH	Size (bp)	Name	Length	GC%	T_m (°C)
5′-GCC CAG GAC TGG TGA AGC-3′	376	VH4/6 outer	18	66.7	65
5′-ACC TGA GGA GAC GGT GAC-3′	376	JH	19	63.2	65.1

Table 1b
Sequences of the allele-specific primers for IgH used in the second round PCR.

Sequences of the allele specific primers for IgH	Size (bp)	Name	Length	GC%	T_m (°C)
5′-ATC TAT TAT AGT GGG AGC ACC-3′	177	VH4/6 inner	21	42.9	57.9
5′-ACC CCG TAC CAG CTG CCT CC-3′	177	JH	20	65	68.6

IgH: heavy chain gene; PCR: polymerase chain reaction; T_m: melting temperature; bp: base pair; VH: variable segments in heavy chain gene; JH: joining segments in heavy chain gene.

of neurological abnormalities (manifestation of increased intracranial tension, convulsion and cranial nerve palsy) and in 33 patients as part of their routine work up at first presentation; 24 patients at first presentation and 9 patients at relapse.

Twenty samples tested for IGHR by light cycler, 3 of them with neurological abnormalities. All specimens tested for IGHR were from B-precursor ALL patients. The remaining 25 samples were excluded, 10 because they were T phenotype and 15 due to poor quality DNA.

The results were compared to classic cytology routinely done for all samples. Medical ethical committee of Assiut University approved this study.

2.1. Flow cytometry

2.1.1. Cell counting and preparation

CSF was centrifuged at 100 × *g* for 10 min within 2 h of obtaining the sample. For flow cytometry analysis, a minimum of 1 ml CSF containing at least one cell/10 μl was needed. Flow cytometry could not be accomplished for samples of insufficient cell numbers (one cell/10 μl of CSF).

The supernatant was discarded and the cell pellet washed with phosphate buffered saline, and then by hemolysate.

Antibody cocktails were added to each tube according to the manufacturer's recommendations. Immunophenotyping was performed by standard three-colour immunofluorescent staining using fluorescence-labeled monoclonal antibodies, directed against the following surface markers [CD45, CD34, Terminal Deoxynucleotidyl Transferase (TdT) and CD33 conjugated with FITC from Caltage laboratories (Austria), CD14, CD19 and CD10 conjugated with PE from BD Pharmingen (Becton Dickinson, Biosciences), CD3 and CD19 conjugated with CyQ from IQ Products (Groningen, Netherlands) CD4/CD8 and kappa/lambda dual colored monoclonal Abs from Becton Dickinson (Biosciences, San Jose, CA)].

The antibody cocktails were selected according to the patients' baseline bone marrow immunophenotyping results and in combinations show atypical pattern of antigen expression. The following mixes were used:

(1) Isotypic control;
(2) CD45/CD14;
(3) CD4/CD8/CD3;
(4) Cyt TdT/CD10/CD19;
(5) κ/λ/CD19;
(6) CD34/CD19;
(7) CD33/CD19.

Mix 6 was used if the BM lymphoblasts in the base line immunophenotyping showed expression of CD34. Mix 7 was used only if the lymphoblasts in the base line BM immunophenotyping showed aberrant myeloid expression.

2.1.2. Flow cytometric analysis

The flow cytometer (FACSCaliber; Becton Dickinson) was calibrated using CALIBRITE-3 beads FACSComp software. Data acquisition and analysis was performed using CellQuest software (Becton Dickinson).

2.1.3. Polymerase chain reaction

DNA extraction from CSF was done by high-pure template kit (Roche Diagnostic, Mannheim, Germany).

2.1.4. Principle

The extracted DNA was analysed for IgH chain gene rearrangements as follows:

First round PCR using consensus primers to amplify variable segments (VH)–joining segments (JH) of IgH gene to obtain sufficient product for second round PCR.

Second round PCR of the first round product using allele specific primers to amplify complementary determining region (CDR) of JH of IgH gene. This was performed to further specify the target gene.

2.1.5. Equipment

Light Cycler Instrument (Roche Diagnostics, Mannheim, Germany).

2.1.6. Reagents

(a) Primers [9] are represented in Tables 1a and 1b.
(b) LightCycler® FastStart DNA Master^{plus} SYBR Green I.

Master mix for IgH first and second round PCR amplification were done as manufacturer instruction.

Amplification was done using thermal cycler. The thermal profile includes:

- Initial denaturation step at 94 °C for 5 min.
- 40 cycles of amplification:
  - denaturation at 94 °C for 30 s.
  - annealing at 56 °C for 30 s.
  - extension at 72 °C for 45 s.

2.1.7. PCR protocol on LightCycler® for SYBR Green detection is represented in Table 2

Quantification program: amplification curves were obtained and the fluorescence values versus cycle number were displayed.

Melting curve program: assessment of the specificity of the amplified product was achieved by performing a melting curve analysis. The resulting melting curve allows discrimination between primer dimer and specific product.

2.1.8. Monitoring PCR with the SYBR Green 1 dye

SYBR Green 1dye binds to the minor groove of dsDNA. Fluorescence is generally enhanced by binding. During the various stages of PCR, different intensities of fluorescence signals can be detected, depending on the amount of dsDNA that is present. All DNA become single stranded after denaturation. At this stage of reaction SYBR Green 1 dye will not bind and the intensity of flouresence signal will be low.

During annealing the PCR primers hybridize to the target sequence, resulting in small parts of dsDNA to which SYBR green 1 dye can bind thereby increasing fluorescence intensity.

In the elongation phase, the PCR primers are extended, and more SYBR Green 1 dye can bind. At the end of the elongation phase all of the DNA has become double stranded and a maximum amount of dye is bound. The fluorescence is recorded at the end of the elongation phase and increasing of amount of PCR product can be monitored from cycle to cycle.

2.1.9. Melting curve analysis of amplicons with SYBR Green 1 detection

Each dsDNA product has its own specific melting temperature (T_m) which is defined as the temperature at which 50% of the DNA becomes single stranded, and 50% remains double stranded, the most important factors that determine that T_m of dsDNA are the length and the GC content of that fragment.

In going from low to high GC content a difference of up to 40 °C can be measured in T_m. Checking the T_m of a PCR product can thus be compared with analyzing a PCR product by length in gel electrophoresis.

3. Results

In this work, 45 CSF-samples were examined. Twenty-one samples were positive by FCM [21/45 (46.6%)]. Fifteen samples [15/45 (33.3%)] could not be analysed because of insufficient cell numbers.

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Table 2
PCR protocol on LightCycler® for SYBR Green detection.

Analysis mode	Cycles	Segment	Temperature	Hold time	Acquisition mode
Pre-incubation					
None	1		95 °C	10 min	None
Amplification					
Quantification	45	Denaturation	95 °C	1 s	None
		Annealing	60 °C	10 s	None
		Extension	72 °C	20 s	Single
Melting curve					
Melting curve	1	Denaturation	95 °C	0 s	None
		Annealing	65 °C	15 s	None
		Melting	95 °C	0 s	Continuous
Cooling					
None	1		40 °C	30 s	None

PCR: polymerase chain reaction.

Twelve samples were positive by IGHR [12/20 (60%)] and only 10 samples were positive by cytology [10/45 (22.2%)]. A total of 26/45 positive samples were detected; eight samples were positive for both FCM and cytology, five samples were positive for both IGHR and cytology (Table 3). Thirteen samples [13/45 (28.9%)] were positive by FCM and negative by cytology and three cases were positive by IGHR and not by cytology or FCM (two could not be analysed due to insufficient cell numbers and one was negative) (Table 4). Two samples were positive for both cytology and FCM and not by IGHR and two cases were positive for cytology but not by FCM (Table 4). The first was negative for all markers by FCM and positive by PCR and the cells in the second were reactive T lymphocytes and negative for malignancy by FCM. Table 5 shows results of 20 patients tested by all three methods.

The gated cells in the FCM analysis vary greatly from 20 to 9808 cells; analysis of cases with large number of cells (Fig. 1) was not difficult as those cases with very low count number that needs aberrant phenotype to ensure the malignant involvement (Fig. 2).

The percentages of positive cases by the three methods in relation to the clinical manifestations are shown in Table 6. All the 15 samples that had no sufficient cells to analyse were from cases at first presentations. There were two patients with CNS manifestation; one showed negative results with cytology and FCM and the other was positive by cytology and negative for malignant infiltration (all the cells were reactive T lymphocytes) by FCM. These two cases diagnosed as toxic encephalitis by MRI.

4. Discussion

Hematological malignancies comprise many prognostically distinct subtypes, thus, a uniform approach to therapy would be inappropriate [10]. Instead, emphasis is placed on a strict assessment of risk at the time of diagnosis, so that only patients at high risk for relapse are treated with more intensive therapy, while at those lower risk may have less toxic treatment. CNS involvement is important for the prognosis and treatment; it requires CNS-directed therapy including irradiation and high dose chemotherapy [11]. Leukemic blasts in CSF can be found in one-third of patients at diagnosis, the majority have no neurological symptoms [12]. Accurate CNS assessment at presentation or relapse is very essential for treatment stratification. Recent treatment protocols offer high dose chemotherapy and CNS radiotherapy only for patients with CNS infiltration and reduce treatment for patients without CNS infiltration to decrease the toxicity of treatment and late effect of systemic chemotherapy and CNS radiotherapy [13].

Until now, no absolute standard has been established to diagnose the involvement of CNS and all methods conventionally applied are associated with problems. All methods used for detection of leptomeningeal seeding including cytology, flow cytometry and the DNA-based examination have pitfalls that need to be considered. Conventional cytology is positive only when large numbers of neoplastic cells are present in the CSF. The interpretation of the results is based on morphology, and therefore, poor fixation or cell

Table 3
Detection of CNS infiltration by IGHR and FCM in comparison to cytology in ALL patients.

	IGHR analysis		Immunophenotyping by FCM		
	Positive	Negative	Positive	No cells	Negative
Cytology					
Positive	5/20	2/20	8/45	0/45	2/45
Negative	7/20	6/20	13/45	15/45	7/45

IGHR: heavy chain gene rearrangements; FCM: flow cytometry.

Table 4
Positive CSF samples for malignant involvement by the three methods in 45 ALL patients.

No.	Clinical manifestation	Cytology	FCM	IGHR
1	CNS (M)	−ve	+ve	ND
3	CNS (M)	+ve	+ve	+ve
5	RW up	−ve	+ve	ND
6	CNS (M)	−ve	+ve	ND
8	RW up	+ve	+ve	+ve
9	CNS (M)	−ve	−ve	+ve
10	CNS (M)	−ve	+ve	ND
11	Relp	−ve	+ve	ND
13	Relp	+ve	+ve	ND
14	Relp	+ve	+ve	+ve
16	CNS (M)	−ve	+ve	ND
22	CNS (M)	+ve	+ve	ND
23	RW up	−ve	No cell	+ve
26	Relp	+ve	+ve	+ve
27	CNS (M)	−ve	+ve	+ve
28	Relp	+ve	+ve	−ve
29	CNS (M)	+ve	−ve	ND
33	RW up	−ve	No cell	+ve
35	RW up	−ve	+ve	+ve
36	RW up	+ve	+ve	−ve
38	CNS (M)	−ve	+ve	ND
40	Relp	−ve	+ve	ND
41	Relp	−ve	+ve	ND
42	CNS (M)	+ve	−ve	+ve
44	Relp	−ve	+ve	+ve
45	Relp	−ve	+ve	+ve

CNS (M): central nervous system manifestation; RW up: routine work up of the ALL patient at first presentation; Relp: Relapsed ALL; FCM: flow cytometry; IGHR: heavy chain gene rearrangements; +ve: positive; −ve: negative; ND: not done; no cell: insufficient cell number.

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Table 5
Results of CSF samples for malignant involvement by the three methods in 20 ALL patients.

No.	Clinical manifestation	Cytology	FCM	IGHR
2	RW up	−ve	−ve	−ve
3	CNS (M)	+ve	+ve	+ve
8	RW up	+ve	+ve	+ve
9	CNS (M)	−ve	−ve	+ve
12	RW up	−ve	No cells	−ve
14	Relp	+ve	+ve	+ve
20	RW up	−ve	No cells	−ve
21	RW up	−ve	No cells	−ve
23	RW up	−ve	No cells	+ve
26	Relp	+ve	+ve	+ve
27	CNS (M)	−ve	+ve	+ve
28	Relp	+ve	+ve	−ve
33	RW up	−ve	No cells	+ve
34	RW up	−ve	−ve	−ve
35	RW up	−ve	+ve	+ve
36	RW up	+ve	+ve	−ve
37	RW up	−ve	No cells	−ve
42	CNS (M)	+ve	−ve	+ve
44	Relp	−ve	+ve	+ve
45	Relp	−ve	+ve	+ve

CNS (M): central nervous system manifestation; RW up: routine work up of the ALL patient at first presentation; Relp: relapsed ALL; FCM: flow cytometry; IGHR: heavy chain gene rearrangements; +ve: positive; −ve: negative; ND: not done; no cells: insufficient cell number.

debris may lead to negative findings at time when the malignant cells are actually present in the CSF. In addition, some examiners may consider atypical morphology as inconclusive evidence for malignancy while others may read it as positive cytology [8]. In our study only 10 cases were positive by cytology, 1 of them was false positive; the cells were reactive as proved by FCM. This indicates that cytology alone cannot assess the CSF infiltration.

Many studies were published about the superiority of FCM in detecting CSF residing abnormal cells as compared to conventional cytomorphology [2,4,5,14–19]. Our data are in accordance with them and show high sensitivity of FCM in detecting malignant cells even in the absence of positive cytomorphology, which were about 29% in our study and between 27 and 78% in the others. In spite of differences as regards conventional cytology performances, all studies clearly showed that FCM is able to increase dramatically the detection of occult CSF infiltration. Taking in concern that the previous studies were based upon analysis of heterogeneous cohorts of cases or focused on lymphomas.

As regards the percentages of positive cases by FCM in relation to the clinical manifestations, we noticed that they were twice those detected by cytology in cases at first presentation, and reached the level reported in the literature [12].

Taken together, our and literature results indicate FCM as the first choice technique, probably due to its intrinsic capability of exploring a large series of cell specificities at a single cell level. It can offer a unique and objective method by the combination of different

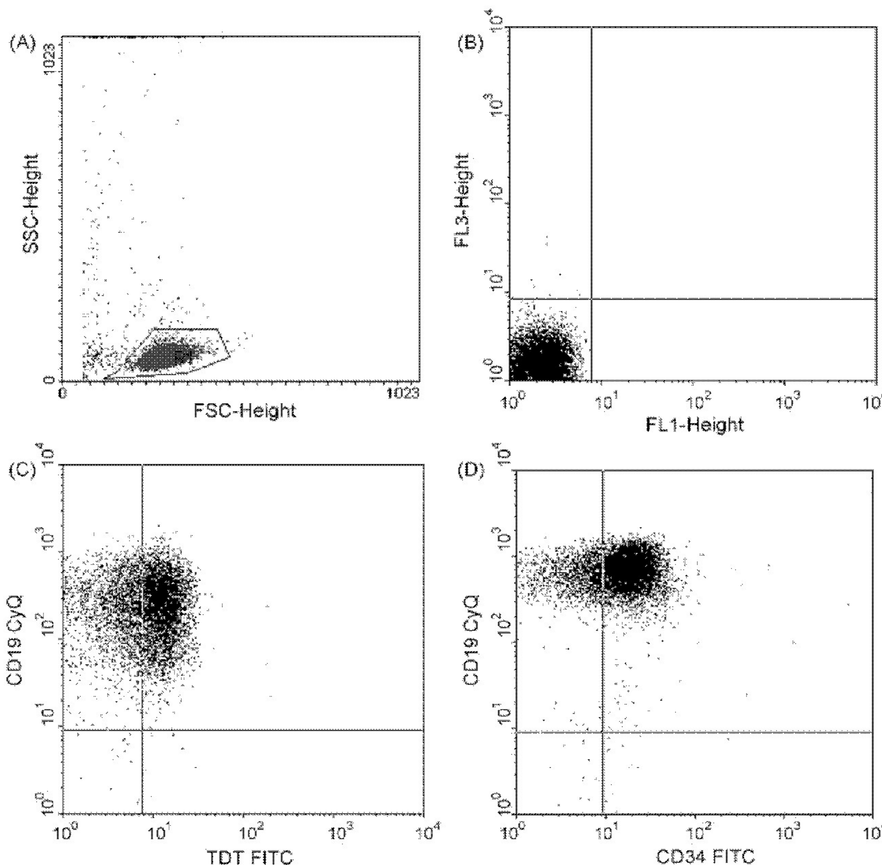

Fig. 1. (A) Dot plot of FSC vs. SSC showing a distinct population (R1); (B) dot plot of FL1 vs. FL3 gated on R1 showing clear isotypic control; (C) dot plot of TdT-FITC vs. CD19-CyQ gated on R1, showing that the gated cells co-express both markers; (D) dot plot of CD34-FITC vs. CD19-CyQ gated on R1 showing that the gated cells express CD34 also.

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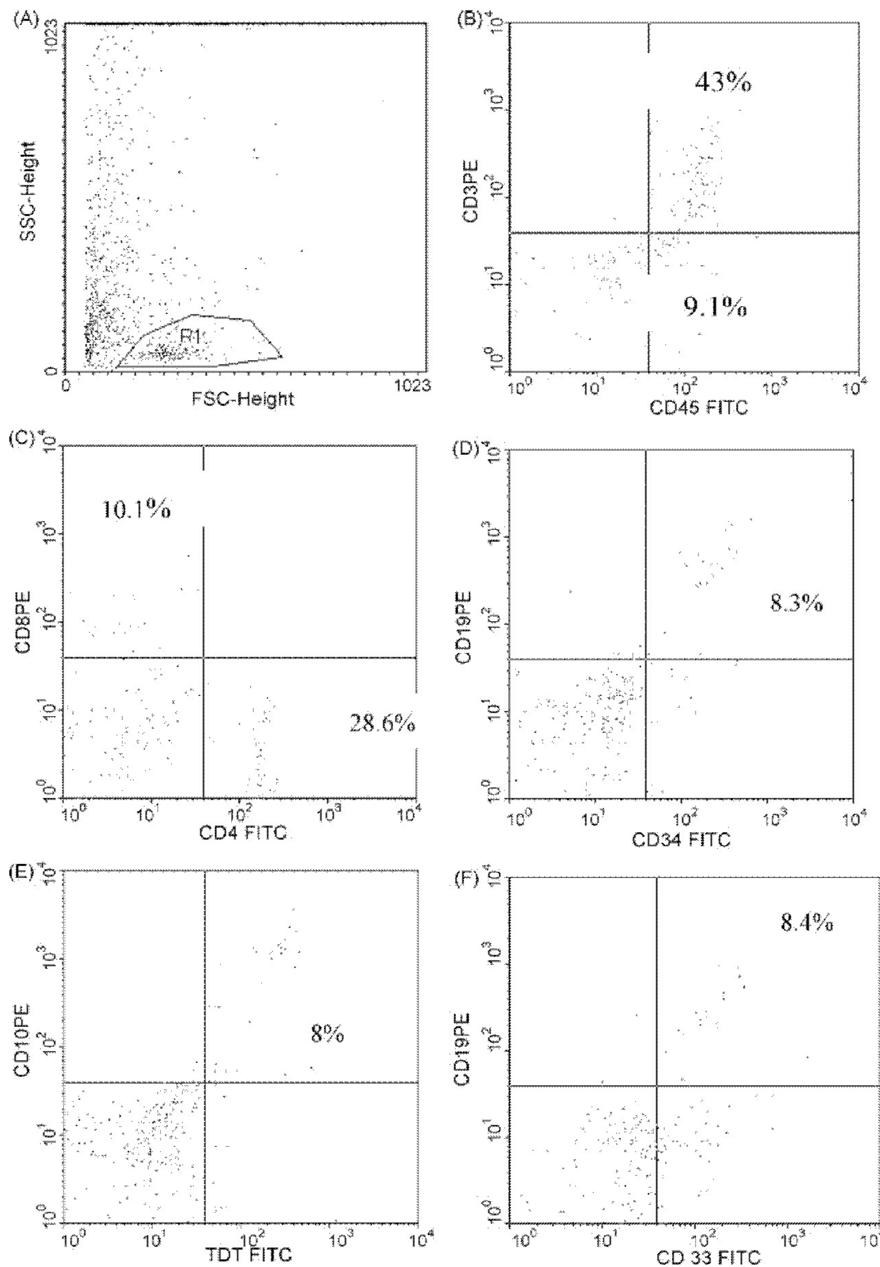

Fig. 2. (A) Dot plot of FSC vs. SSC showing a distinct population (R1); (B) dot plot of CD45-FITC vs. CD3-PE gated on R1 showing two population groups the first is of T lymphocytes (43%) that co-express CD45 and CD3 and the second is of malignant population that express CD45 and not CD3 (9.1%); (C) dot plot of CD4-FITC vs. CD8-PE gated on R1 showing that the T lymphocytes are reactive and distributed into CD4 cells (28.6%) and CD8 cells (10.1%); (D) dot plot of CD34-FITC vs. CD19-PE gated on R1 showing that 8.3% of gated cells co-express both markers (lymphoblast cells) which are the malignant population; (E) dot plot of CDTdT-FITC vs. CD10-PE gated on R1 showing that 8% of the gated cells co-express both markers (lymphoblast cells) which are the same malignant population; (F) dot plot of CD33-FITC vs. CD19-PE gated on R1 showing that the same population 8.4% of the gated cells express aberrant myeloid marker.

strategies as aberrant immunophenotype [15]. It is a simple, quick and reliable technique. Results were available within 2 h, which can speed up the therapeutic management; this may be a cost-reducing factor [18]. One of the strengths of FCM is its sensitivity. In our results, CSF involvement could be demonstrated by FCM and not by cytology in cases with very low cell counts that accounted for only 0.86% of the total number of cells. This is similar to the 1.4% reported by French et al. [17] and 0.9% reported by Finn et al. [16]. This can be attributed to the simultaneous evaluation of multiple surface markers on each cell.

However, FCM could still be falsely negative in some cases that can be detected by other methods. This may be explained by loss of some antigens in cases of relapsed ALL or dilution of malignant B cells by large numbers of polyclonal normal B-lymphocytes [14]. It

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Table 6
Distribution of the CSF-positive cases according to the clinical manifestation in ALL patients.

	Cases at presentation	Relapsed cases	CNS manifestation	Total
Cytology	2/24 (8.33%)	4/9 (44.44%)	4/12 (33.33%)	10/45 (22.2%)
FCM	4/24 (16.66%)	9/9 (100%)	8/12 (66.66%)	21/45 (46.6%)
IGHR	4/11 (36.36%)	4/5 (80%)	4/4 (100%)	12/20 (60%)

CNS: central nervous system; IGHR: heavy chain gene rearrangements; FCM: flow cytometry.

is also important to recognize, that while FCM could detect 13 cases of CSF infiltration with negative cytology, a total of 15 cases (33.3%) could not be analysed due to insufficient number of cells. This figure is slightly higher than that reported by French et al. [17] (29%). This may be due to a higher number of cases at first presentation in relation to relapsed and symptomatic ones in our study.

The DNA-based molecular techniques do not require intact cells. DNA is stable and can be recovered from CSF even after tumor cell lysis, probably making it a more sensitive indicator of malignancy than FCM and cytology requiring presence of intact tumor cells [20]. The detection of clonal Ig gene rearrangements using PCR technique offers an alternative because of its high sensitivity [21,22]. The rearrangement of variable, diversity and joining segments (VDJ) of IgH genes generates unique DNA junctional sequences that are specific by its size and sequence to each B cell clone [23].

Many attempts have been made to use PCR for identifying IGHR in cells from CSF [8,20,21,24–26]. Several factors contribute to this diversity, including the presence of somatic hypermutations that prevent binding of the PCR primers and the fact that the consensus primers for any particular assay are not complementary to all V regions. Finally, as with all PCR protocols, false positive results due to technical problems as contamination are possible [20].

Some authors applied the highly sensitive semi-nested PCR [27] in the search for IgH gene rearrangements in CSF sample cells. This technique has proved to be sufficiently sensitive to produce a detectable band from a single B cell [24]. However to achieve a reliable detection of monoclonality, previous methods require the presence of approximately >1% of clonal B cells to show a clearly visible rearranged band [28]. The sensitivity of PCR methods for Ig gene rearrangement is limited by the separating power of the gel and the discriminating power of the eye to recognize a faint band of clonal B cell superimposed upon a diffuse smear, which is generated by the reactive polyclonal B cell population present in all tissues [29].

Real-time PCR permits accurate detection of PCR products during the exponential phase of the PCR amplification process, which is in full contrast to the classical PCR end point detection. Owing to the real-time detection of fluorescent signals during or after each subsequent PCR cycle, detection of PCR data can be obtained in a short time and no post-PCR processing is needed, thereby drastically reducing the risk of PCR product contamination [30].

Few studies have compared the sensitivity of IGHR with morphological analysis of CSF; all of them on lymphoma and no one by real-time. Only one showed that IGHR analysis did not appear to be higher sensitive than morphological evaluation, in which a low incidence of lymphomatous spread to spinal fluid was found (8 from 76 patients) [25]. Possibly, because of pretreatment of the majority of patients with steroids prior to lumbar puncture and the small CSF volume analyzed [22].

Our results showed greater CSF involvement as detected by real-time PCR than cytology (60% vs. 22.2%). This agree with the results of Scrideli et al. [31], who reported molecular CSF involvement greater than observed by morphologic criteria (45.9% vs. 5.4%) and the 4-year event-free survival was lower in the group with molecularly detected CSF involvement ($p = 0.01$). However, still the incidence is higher in our series than his, which might be because they examined newly diagnosed ALL while in this study we included relapsed patients as well.

PCR analysis of the IgH gene typically involves the use of a consensus primer pair, with the upstream primer being homologus to a V segment and the downstream primer annealing to one of the J segments [32]. Usually a single J region primer is sufficient to recognize all six possible J segments, but no single V region primer recognizes all V segments, since there are many more V segments that are more heterozygous as compared with J segments. This is the primary explanation for the lack of a 100% diagnostic sensitivity of a single primer pair IgH PCR assay [33].

There were two cases (10%) positive for cytology and FCM and negative by real-time PCR. False negative results in PCR were reported in small samples, and in the presence of a low cell count. False positive results in real-time PCR could be eliminated by optimization conditions. The design of clone-specific primers and the annealing temperature are important steps for achieving accurate data. Melting curve analysis was found to be an essential tool for characterizing the PCR products. However, since a true gold standard does not exist and no follow-up data are available there is no way of proving that positives were true and not false positive. Thus, sensitivity, specificity and predictive value of positive or negative results of IgH gene rearrangement by real-time PCR in the CSF require further evaluation.

In conclusions: the diagnostic value of FCM and IGHR are two to three times more than that of cytology. Malignant cells in CSF can be classified according to the immunological surface profile by FCM. Therefore, immunophenotyping by FCM is recommended for routine diagnosis of CSF infiltration combined with cytology to increase the diagnostic yield. Furthermore, IGHR analysis by real-time PCR appears to be a useful addition to morphological and FCM analysis of CSF in the evaluation of CNS infiltration in ALL. It is reliable; relatively sensitive and highly recommended if there are no sufficient cell numbers for FCM analysis in cases at presentation, in relapsed cases or in those with CNS manifestations with negative results for both cytology and FCM.

Acknowledgments

Contributions. DS designed, performed the flow cytometry work, analyzed data and wrote the paper; HB designed, performed the real-time PCR and contribute in writing the paper; AMA involved in care of patients, sample procurement and collected clinical data; SS designed the research and collected the samples.

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