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Ten-year Experiences on Initial Genetic Examination in Childhood Acute Lymphoblastic Leukaemia in Hungary (1993–2002). Technical Approaches and Clinical Implementation

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Abstract A nationwide study was started in 1993 to provide genetic diagnosis for all newly diagnosed childhood ALL cases in Hungary using cytogenetic examination, DNA-index determination, FISH (aneuploidy, ABL/BCR, TEL/AML1) and molecular genetic tests (ABL/BCR, MLL/AF4, TEL/AML1). Aim of the study was to assess the usefullness of different genetic methods, to study the frequency of various aberrations and their prognostic significance. Results were synthesized for genetic subgrouping of patients. To assess the prognostic value of genetic aberrations overall and event-free survival of genetic subgroups were compared using Kaplan-Meier method. Prognostic role of aberrations was investigated by multivariate analysis (Cox's regression) as well in comparison with other factors (age, sex, major congenital abnormalities, initial WBC, therapy, immunophenotype). Five hundred eighty-eight ALL cases were diagnosed between 1993–2002. Cytogenetic examination was performed in 537 (91%) (success rate 73%), DNA-index in 265 (45%), FISH in 74 (13%), TEL/AML1 RT-PCR in 219 (37%) cases producing genetic diagnosis in 457 patients (78%). Proportion of subgroups with good prognosis in prae-B-cell ALL was lower than expected: hyperdiploidB 18% (73/400), TEL/AML1+ 9% (36/400). Univariate analysis showed significantly better 5-year EFS in TEL/AML1+ (82%) and hyperdiploidB cases (78%) than in tetraploid (44%) or pseudodiploid (52%) subgroups. By multivariate analysis main negative prognostic factors were: congenital abnormalities, high WBC, delay in therapy, specific translocations. Conclusion: Complementary use of each of genetic methods used is necessary for reliable genetic diagnosis according to the algorythm presented. Specific genetic alterations proved to be of prognostic significance.

Keywords ALL · Childhood · Chromosome aberration · FISH · Prognostic factor

Abbreviations

ALL	acute lymphocytic leukaemia
EFS	event free survival
FISH	fluorescens in situ hybridization
MLL	mixed linear leukaemia gene
PCR	plymerase chain reaction
WBC	white blood cell

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Introduction

Current intensive chemotherapy cures near 80% of children with acute lymphoid leukaemia (ALL) [5, 6, 22, 25, 29, 30]. Beside impressive advances in survival rates of patients in the latest decades a significant percentage of cases die due to resistant leukaemia, relapse and/or severe early toxicity of combined chemotherapy. Many patients suffer from late side effects of chemo- or radiotherapy which have major influence on their quality of life [5, 6, 22, 25, 29, 30]. In order to further improve therapeutic results and reduce therapy-related toxicity in childhood ALL we have to determine prognosis at the time of diagnosis and provide tailored therapy for individual cases [5, 6, 22, 23, 25]. This means to moderate chemotherapy and minimize side effects in favourable subgroups and to intensify only for cases with a poor prognosis. In addition to commonly used clinical, hematological and immunological prognostic factors initial karyotype has been proved to have independent prognostic value in childhood ALL [5, 6, 9, 21–25, 29, 30, 32]. Although genetic aberrations in leukaemic cells are accepted to underlie leukaemogenesis and determine disease characteristics only specific translocations with the worst prognosis (ABL/BCR, MLL/AF4) are used as determinants for the choice of therapy. Even if hyperdiploidy or the presence of TEL/AML1 rearrangement confers the best prognosis they are not routinely included as prognostic determinants in therapeutic protocols [5, 6, 9, 21, 23, 24, 26, 29, 30, 32]. These patients are classified together with karyotypically normal cases according to routine parameters (age, initial white blood cell count, prednisolone-response). There is no specific "low risk protocol" (e.g. without anthracyclins) for high hyperdiploid and TEL/AML1-positive patients.

A prospective nationwide population-based study was started in 1993 in Hungary with the aim to provide genetic diagnosis for all newly diagnosed childhood ALL cases. New genetic methods were introduced to complete cytogenetic examination: flow cytometric DNA-index determination, FISH for numerical aberrations and translocations, molecular genetic tests for ABL/BCR, MLL/AF4, TEL/AML1 gene rearrangements. Results of all the tests were synthetized into a genetic subgrouping for childhood ALL. Main questions of the study were as follows:

Are the methods used for the genetic screening of initially diagnosed childhood ALL patients in Hungary reliable for prognostic classification?

How can we synthesize results of different genetic tests in subgrouping of patients?

Can we prove the prognostic significance of different genetic alterations?

What kind of algorythm can we suggest for the genetic classification of patients?

Can this prognostic classification be considered as a basis for the choice of therapy?

How to go on? What changes can we recommend to further improve the quality of genetic diagnosis for ALL patients in Hungary?

Hereby we describe the frequency of different genetic aberrations and our experience with genetic testing in childhood ALL. We analyse survival of patients in various genetic subgroups in comparison to other prognostic factors.

Patients

Diagnosis and treatment of children with haematologic malignancies in Hungary have been performed for more than three decades in ten haematooncological centres of the Hungarian Paediatric Oncology Network established in 1971 [31]. This work has been based on standardized diagnostic and therapeutic principles in accordance with international protocols. The coordination of the network, confirmation of diagnosis and population-based registration of cases have been carried out by the II. Department of Pediatrics, Semmelweis University, Budapest. This network covers the whole country and all children with leukaemia in Hungary are referred to one of the haematooncological centres for final diagnosis and specific therapy.

All ten centres of the Hungarian Paediatric Oncology Network and diagnostic laboratories agreed to participate in our study. This cooperation included intention to perform genetic tests by all newly diagnosed ALL cases and reporting genetic results to the central registry for genetic subgrouping. Diagnosis of ALL in all cases was based on standardized principles in one of the ten centres, followed by registration and confirmation by the coordinating centre in Budapest (central verification of diagnosis and remission: initial blood and bone marrow smears, day 33 bone marrow). Haematological and immunological analyses, DNA-index determination were carried out mainly in the local laboratories of treating centres, university hospitals; for centres in Budapest in two centralized laboratories. Cytogenetic tests were performed in local genetic laboratories affiliated to hematooncologic centres, in Budapest one central laboratory according to standardized protocol. To peripheral centres where no genetic laboratory was available we suggested sample transfer to the nearest university laboratory (Budapest, Pécs, Debrecen). FISH and molecular genetic tests were introduced in university centres most experienced in genetic and immunologic studies and performed for the whole country. Technical, financial and realibility reasons lead to fast centralization of immunophenotyping and genetic diagnosis after the early years of the study (Ist Institute of Pathology of University of Budapest; Institute of Pathology of University of Pécs; Institutes of Molecular Pathology and

Clinical Genetic Center, Department of Paediatrics of University of Debrecen).

ALL cases were treated in one of the centres of the Network according to international protocols recommended by the Berlin-Frankfurt-Münster (BFM) Study Group (ALL-BFM 90: 185 cases diagnosed January 1st 1993-December 31st; ALL-BFM 1995: 394 cases diagnosed January 1st 1996-October 31st 2002; ALL-IC 2002: nine cases diagnosed after 1 November, 2002) [29, 30]. Patients were stratified as part of their therapeutic protocol according to BFM risk factors (standard, medium, high risk groups). Mature B-cell leukaemia patients were treated according to the NHL-BFM 90 and 95 protocols in the same time periods.

Methods

The major goal of the study was to identify most important genetic aberrations of prognostic value. For this purpose initial routine cytogenetic examination, in addition flow cytometric DNA-index determination, FISH (for numerical aberrations, ABL/BCR, TEL/AML1, gene rearrangements) and molecular genetic tests (ABL/BCR, MLL/AF4, TEL/AML1, immunoglobuline chain and T-cell receptor gene rearrangements) were applied. The following strategy was recommended: to identify patients with hyperdiploid karyotype and those with specific translocations: cytogenetic and flow cytometric analysis were performed simultaneously at diagnosis. If DNA index was above 1.16 (hyperdiploid B) or between 1.05–1.16 (hyperdiploid A) and/or chromosome examination failed to identify supernumerary chromosomes, FISH technique was applied using centromere-specific probes for the chromosomes most commonly involved (4, 6, 10, 17, 18, 21, 22, X) [1, 3, 4, 7, 10, 27, 28, 33]. Based on our earlier experience this spectrum was completed with probes for chromosome 8, 11 and 13 [19]. If DNA-index was diploid (0.97–1.05) and chromosome examination normal/unsuccessful, molecular genetic test (RT-PCR) was recommended for the detection of the cryptic translocation 12;21. If routine karyotyping failed only molecular genetic tests were able to detect specific translocations (ABL/BCR, MLL/AF4, TEL/AML1) [1, 3, 4, 7, 10, 27, 28, 33].

Cytogenetic Analysis

Unstimulated isolated bone marrow and/or peripheral blood cells were cultured for 24 h. Metaphase chromosomes were prepared according to standard procedures (G-banding). Minimum of ten metaphases were evaluated if available [21]. In evaluating karyotypic changes, the International System for Human Cytogenetic Nomenclature (ISCN) was applied [12].

Flow Cytometric DNA-Index Determination

For the analysis of DNA content flow cytometry was performed according to standard procedures using peripheral lymphocytes as control. A DNA-index of lower than 0,97 was considered hypodiploidy, while that above 1,05 meant hyperdiploidy A, above 1.16 as hyperdiploid B [11, 33].

Fluorescence in situ Hybridization (FISH)

Numerical chromosome abnormalities were analyzed in interphase lymphoblast cells with centromere-specific probes (4, 6, 8, 10, 12, 13/21, 15, 17, 18, 20, 22, X) according to standard methods described by Berger et al. [1, 4]. Although on a subset of patients a total panel was applied, FISH was usually used mainly for the identification of the most common supernumery chromosomes (8, 17, 18, 21, X). During the last years of the study FISH was also used for the identification of ABL/BCR, TEL/AML1 and MLL genes' rearrangements.

Molecular Genetic Tests

TEL/AML1 rearrangement was tested by RT-PCR, ABL/BCR, MLL, IgH, κ, λ, TCR gene rearrangements by standard PCR procedures [3, 7], not only for the determination of different genetic subgroups of ALL but also to serve as a follow up test for minimal residual disease.

Data Collection and Analysis

Clinical, haematological, immunological and genetic data were collected by the genetic working team of the Department of Paediatrics, University of Debrecen and the Hungarian Paediatric Cancer Registry, IInd Department of Paediatrics, Semmelweis University, Budapest. For genetic subgrouping of cases results of all available genetic tests were taken into account. By in cases of contradictory results, DNA-index was decisive.

Genetic subgroups were defined as follows:

- HyperdiploidA: chromosome number between 47–50, DNA-index 1.06–1.16
- HyperdiploidB: chromosome number 51 or above, DNA-index ≥ 1.16
- Tetraploid (near-tetraploidy): chromosome number between 92 (±5), DNA-index 2.0 (±0,15)
- Hypodiploid: chromosome number below 46, DNA-index <0,97
- Pseudodiploid: presence of specific translocations or deletions
- Normal: chromosome number 46, DNA-index :0,97–1,05

- Normal, TEL/AML1-positive: chromosome number 46, DNA-index 0.97–1.05, TEL/AML1-positive by RT-PCR or FISH
- Unknown: no available informative genetic test result, testing unsuccessful or not carried out

Population-based data on cases diagnosed between January 1, 1993 and December 31, 2002 and follow-up information were provided by treating centres and the Hungarian Paediatric Cancer Registry with closing date January 31st 2007.

Statistical analysis was performed by the Statistica 6.0 programme package. Event-free and overall survival of patients was estimated by the Kaplan-Meier method. Survival of patients in genetic subgroups was compared by the log-rank test. In addition we examined the prognostic role of different other factors in univariate analysis by the log-rank test (sex, age at diagnosis, time period of diagnosis, pre-existent diseases, immunphenotype, CALLA positivity, coexpression of myeloid markers, steroid pretreatment, therapeutic protocol). To compare the significance of different prognostic factors we performed multivariate analysis (Cox's regression) taking into account results of genetic tests and all commonly used clinical prognostic indicators (age, sex, major congenital abnormalities, initial white blood cell count, time period of diagnosis, delay in therapy, immunophenotype).

Results

Patients

Five hundred eighty-eight new cases of childhood ALL were diagnosed in Hungary between 1993–2002 (total population: 10 million). There were 338 boys (57.5%) and 250 girls (42.5%). Yearly number of new cases ranged from 37 to 77 with an average of 59. As all leukaemic children under the age of 15 are treated at centres of the Hungarian Paediatric Oncology Network this study is representative in the age group of 0–14 yrs old for the whole country. Age-standardized annual incidence of ALL ranged between 23,1–45.5/1,000,000 person years with some yearly variation, with an average of 33.7/1,000,000 person years. Although in the age group of 15–18 yrs old cases are preferably referred to paediatric centres, no population-based data are avilable.

Absolute and relative distribution of cases according to age, initial WBC, congenital disorders, time period of diagnosis and BFM risk groups is given in Table 1. 5 year event-free survival estimated by the Kaplan-Meier method was compared in each subgroup defined by different values or categories of a single clinical parameter. A highly significant difference was observed by univariate analysis (log-rank test) for the different age groups at time of diagnosis, initial WBC, congenital disorders, risk groups (also for immunophenotypes, CALLA status, prednisone-response and delay/refuse of therapy, not in table).

Chromosome Analysis

The yearly change of performance of different types of genetic tests is given in Fig. 1. Frequency of cytogenetic examinations was initially above 80% and increased during the 10 years to near 100%. Success rate showed a similar increasing tendency from around 50% to above 80%. In total 537 out of 588 cases were examined (91%) with an average success rate of 73%. Unfortunately the proportion of aberrant karyotypes remained behind the expected value (average: 39%, literature: 70%) [2, 6, 9, 10, 13, 19, 20, 24, 26, 32], resulting in only 152 out of 588 patients with informative cytogenetic study. The reasons for this can be the failure of recognition of subtle changes due to technical problems like low number and poor quality of metaphases; or unintentional preference to normal, better quality metaphases. To reduce heterogeneity of the quality of testing we introduced regular consultations with the participation of all cytogeneticists. At the same time some geographical differences in the incidence of aberrations cannot be excluded, as we observed in other haematologic diseases (secundary aberrations in CML), which may be related to differences in etiological factors.

Flow Cytometry, FISH, Molecular Genetic Tests

Although in the mid-nineties there was a steep increase in the frequency of flow cytometric *DNA-index* determination up to above 70% of patients, due to a subsequent fall it was performed in total 265/588 cases (45%), yearly average only in 47%. Yearly ratio depended—besides some financial aspects—on the number and distribution of newly diagnosed patients among the treating centres and their access to genetic tests.

FISH was done in 74/588 cases (13%), on average 14,4%. Application of this method was restricted by its high cost. Introduction of molecular genetic tests into ALL diagnostics and widespread use of DNA-index determination was stimulated by the present study in Hungary. During these 10 years percentage of cases tested increased steeply up to 70% for TEL/AML1 RT-PCR and 80% for other molecular genetic tests (ABL/BCR, Ig/TCR gene rearrangement for MRD detection). Altogether 219 patients (37% of all cases) had TEL/AML1 testing, 46 were positive (21%) and 173 negative (79%). Molecular genetic testing was done in a total of 40% of patients.

Table 1 Absolute and relative distribution of ALL cases (N=588, 1993–2002) according to age at diagnosis, initial white blood cell count, congenital disorders, time period of diagnosis and BFM risk groups. Five year event-free survival estimated by the Kaplan-Meier method is compared in each subgroup defined by different values/ categories of a single clinical parameter by the log-rank test, p values considered significant <0,05

Parameters	Subgroups					
Age at diagnosis (years) p: 0,0000	**Infants**: 20 (3,4%)	**1–5**: 312 (53,1%)	**6–9**: 127 (21,6%)	**10–18**: 129 (21,9%)		
5 yr-EFS:	22,9%	75,6%	64,0%	61,2%		
Initial WBC (G/l) p: 0,00001	**<3**: 87 (14,8%)	**3–13,9**: 240 (40,8%)	**14–19,9**: 41 (7,0%)	**20–49,9**: 99 (16,8%)	**50–200**: 79 (13,4%)	**200<**: 42 (7,1%)
5 yr-EFS:	65,1%	74,4%	79,7%	70,3%	57,2%	45,2%
Congenital disorders p: 0,0002	**Down syndrome**: 11 cases (1,9%)	**Other (heart, complex)**: 13 cases (2,2%)	**None**: 565 cases (96,1%)			
5 yr-EFS:	44,6%	38,5%	69,5%			
Time period of diagnosis p: 0,042	**1993–1995**: 184 (31,3%)	**1996–1999**: 257 (43,7%)	**2000–2002**: 147 (25,0%)			
5 yr-EFS:	66,8%	65,2%	78,1%			
Risk groups p: 0,0000	**Standard**: 147 (25,0%)	**Medium**: 340 (57,8%)	**High**: 101 (17,2%)			
5 yr-EFS:	86%	71%	36%			

Genetic Subgrouping of Patients

Taking into account the results of all genetic tests we came to a genetic diagnosis in 457/588 cases (78%). As clinical/ immunological subtypes of ALL have different biologic characteristics in close correlation with underlying genetic aberrations we give descriptive results separately for infants (20 patients), mature-B ALL (22 pts), T-ALL (97 pts) and B-cell precursor ALL (400 pts). In 23 cases immunophenotype was not known, mainly because of unsuccessful or uniformative testing at the early period of the study. Distribution of genetic subgroups, immunophenotypes of ALL and their correlation is given in details in Table 2.

To evaluate the prognostic significance of single aberrations another subgroup of non-T-non-B ALL patients was formed including in addition to prae-B cell patients (400), biphenotypic ALL (20 pts) and 0-cell (6 pts) cases (n=426).

Out of the *20 infant ALL* cases 12 had uninformative genetic tests and eight cases were pseudodiploid (40%) with structural aberrations detected by routine karyotyping (three cases had t(4;11), one 11q23 deletion, one variant translocation 11;19; one case t(1;19), two cases 1p+). Survival was generally poor (4-yr-overall survival 42%, standard error 12%). Unfavourable prognostic factors were as follows: male sex (p=0,02), young age at diagnosis (<6 months, p=0,004), but not the presence or type of structural genetic aberrations (p=0,44).

In the *T-cell group* of 97 patients 22 had uninformative genetic testing, 49 were normal and 13 patients had different specific aberrations (pseudodiploid, 13%). Other types of genetic alterations proved to be rare: seven hyperdiploid A, five hyperdiploid B and 1 hypodiploid. In all cases when carried out (36), TEL/AML1 test was negative. Overall 5-yr survival of cases was 64% (SE 5%), slightly better for boys (70 patients, 69%) than in girls (27 patients, 51%, p=0.053). We could not detect any significant difference between genetic subgroups.

In 22 cases of *mature-B cell ALL* 13 had specific structural aberrations (59%), 4 were normal, 4 "unknown" and one hyperdiploid A. Nine patients had negative and 2 positive TEL/AML1 test (1 pseudodiploid, 1 unknown). Overall 5-year survival of patients was 50% (SE 11%); prognosis did not differ significantly in the subgroup with specific aberrations (pseudodiploid: 46%—all others: 56%, p=0.27).

In the *non-T non-B ALL* patients (in total 426) overall 5-year survival was 79% (SE 2%), event-free survival 73% (SE 2%). We examined the prognostic role of structural

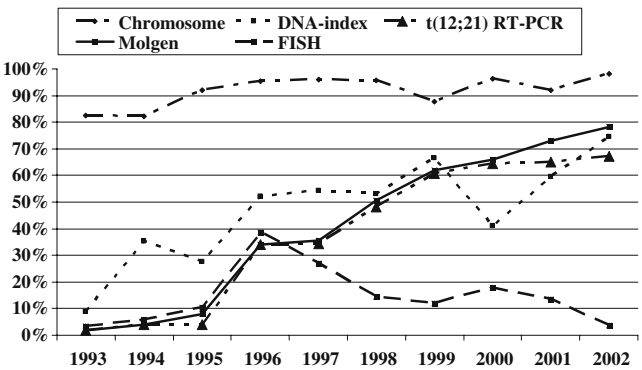


Fig. 1 Percentage of cases studied by different genetic methods: cytogenetic examination, flow cytometric DNA-index determination, FISH, RT-PCR for TEL/AML1 and molecular genetic tests by year of diagnosis (1993–2002)

Table 2 Absolute distribution of ALL cases (N=588, 1993–2002) according to results of all genetic tests synthesized in genetic classification in different clinical-immunphenotypic subgroups. Percentage of cases with genetic aberrations in those succesfully tested

Genet./Immun	Un-Known	Normal; TEL/AML1-	Hypo-diploid	Hyper-diploidA	Hyper-diploidB	Normal, TEL/AML1+	Tetra-ploid	Pseudo-diploid	Total	Total succes.	Ratio of aberrant cases	
0-cell	1	3	–	–	1	–	–	1	6	5	**2/5**	**40%**
Infant	3	9	–	–	–	–	–	8	20	17	**8/17**	**47%**
B-precursor	93	131	8	21	73	36	6	32	400	307	**176/307**	**57%**
Mature B	1	5	1	1	–	1	–	13	22	21	**16/21**	**76%**
Biphenotypic	2	11	–	–	1	1	–	5	20	18	**7/18**	**39%**
T-cell	22	49	1	7	5	–	–	13	97	75	**26/75**	**35%**
Not inform.	9	8	1	2	1	–	–	2	23	14	**6/14**	**43%**
All	131	216	11	31	81	38	6	74	588	457	**243/457**	**53%**

aberrations in 12 cases with Philadelphia translocation, seven cases with 11q aberration, 20 cases with other different structural aberrations and 42 TEL/AML1-positive patients against all other patients (345) without detected structural abnormalities. We came to highly significant differences both in overall survival (p=0.0018) and event-free survival (p=0.00032) (Fig. 2).

Neither the few *0-cell* (n=6) nor *biphenotypic ALL* cases (n=20) form characteristically different groups in comparison to prae-B cell ALL cases concerning clinical parameters or survival, only age at diagnosis was more often above 6 years (50%) in biphenotypic ALL. Concerning genetic alterations, in byphenotypic ALL cases high hyperdiploidy and TEL/AML1 positivity proved to be rare (only a single case each) while pseudodiploidy more frequent (five cases including two Philadelphia-positive cases).

The genetic classification in the group of 400 *prae-B-cell ALL* patients came to the following results (Fig. 3). Twenty-three percent of cases did not have informative genetic testing (unknown subgroup), containing mainly cases diagnosed in the first years of the study when the availability and success rate of genetic tests was the lowest. One third of these cases belonged to the normal subgroup and about 40% was negatively tested for TEL/AML1. Favourable prognostic subgroups: hyperdiploid B (18%) and those cytogenetically normal, TEL/AML1-positive (9%) gave altogether 27% of all cases, 35.5% of those succesfully tested. The pseudodiploid subgroup (8%) contained cases with translocations (nine Philadelphia-positive, six 11q aberrations) or deletions of chromosomal regions frequently involved in ALL. The hyperdiploid A (5%) hypodiploid (2%) and tetraploid (1.5%) subgroups counted the least cases.

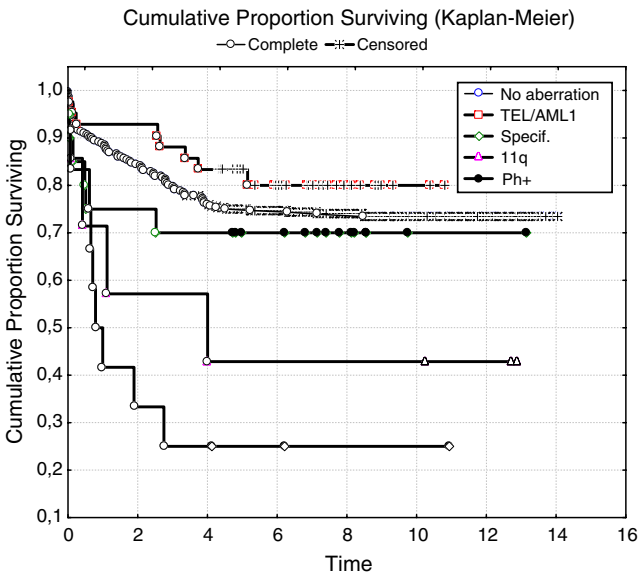
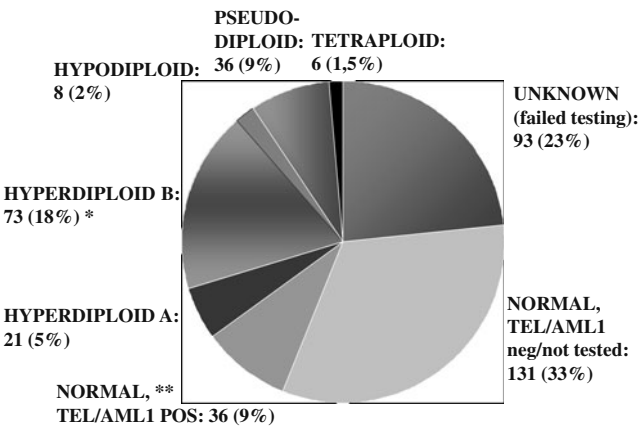
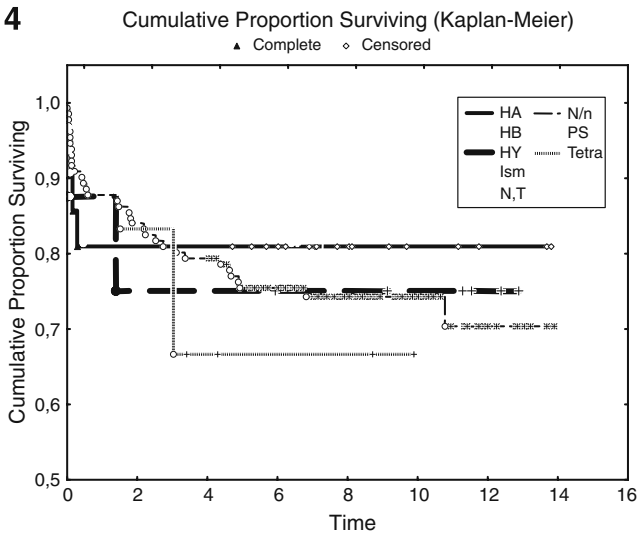


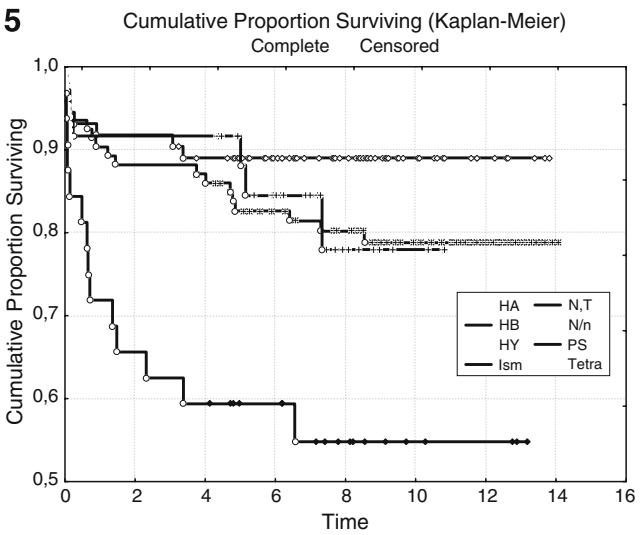
Fig. 2 Comparison of 5-year overall survival estimated by the Kaplan-Meier method in nonT-nonB ALL (400 patients) in cases with different prognostic aberrations (Philadelphia chromosome, 11q aberrations, other structural aberrations, TEL/AML1 rearrangement positive) to cases without these aberrations detected (N)



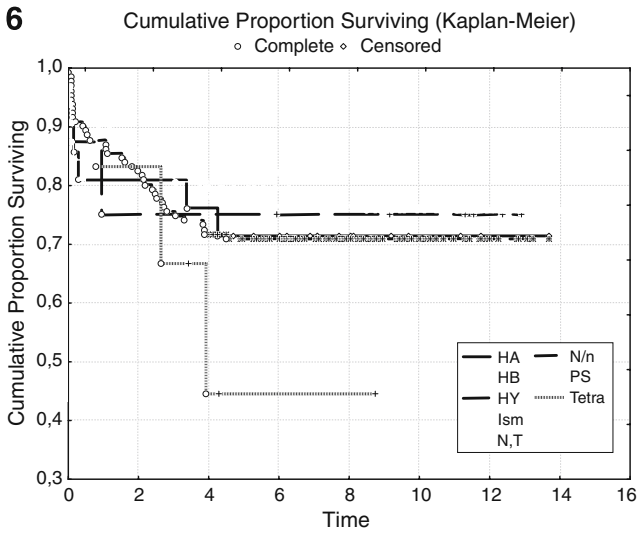
Cases with favourable prognosis (groups*): 109/307 (35,5%)**

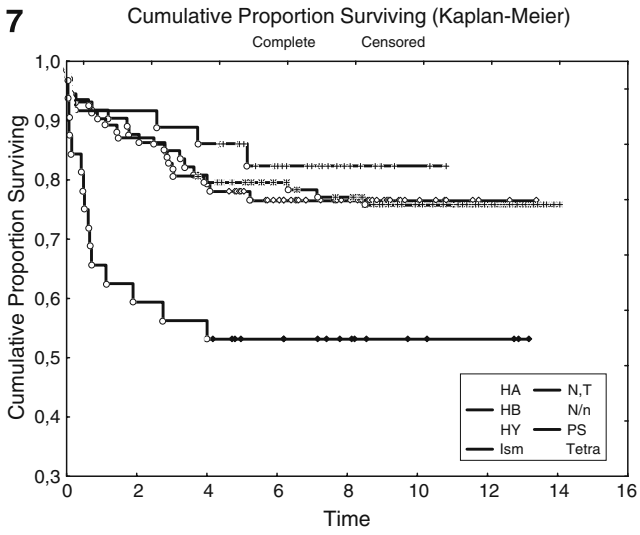
Fig. 3 Relative distribution of genetic subgroups of B-cell precursor ALL patients (N=400, 1993–2002) determined on the basis of results of all genetic tests performed





Figs. 4–5 Comparison of 5-year overall survival estimated by the Kaplan-Meier method in 4-4 genetic subgroups (hyperdiploid A, normal, hypodiploid and tetraploid) (normal, TEL/AML1+, hyperdiploid B, "unknown", pseudodiploid)





Figs. 6–7 Comparison of 5-year event-free survival estimated by the Kaplan-Meier method in 4-4 genetic subgroups (hyperdiploid A, normal, hypodiploid and tetraploid) (normal, TEL/AML1+, hyperdiploidB, "unknown", pseudodiploid)

Kaplan-Meier analysis showed overall 5-year survival of 80% (SE 2%), and event-free survival of 74% (SE 2%) for prae-B-cell patients (Figs. 4–5, 6–7). Concerning the eight genetic subgroups a significant difference (p=0,02) in overall survival could be detected by the log-rank test (univariate analysis) with the best prognosis in normal, TEL/AML1-positive (92%) and hyperdiploid B (89%) patients while the poorest values in pseudodiploid (59%) and tetraploid (67%) cases. Hyperdiploid A (81%) and hypodiploid (75%) patients did not differ much from the unknown (82.5%) and normal (75%) cases. Five-year event-free survival was found to be the best in the normal and, TEL/AML1-positive subgroup (86%), the worst in tetraploid (44%) and pseudodiploid (53%) patients, while the others showed very similar curves (p=0.06). Survival of hyperdiploid B-unknown and the hyperdiploid A-normal subgroups proved to be similar.

Multivariate analysis (Cox's regression) of independent parameters identified congenital abnormalities, infancy, high initial WBC, delay/refuse in therapy, poor prednisone response and specific translocations as main negative prognostic factors. Prognostic effect of commonly used risk group determinants (age, initial WBC, prednisone response, Philadelphia, 4;11 translocations) compared to the genetic subgrouping showed the highest significance for the combined use of clinical hematological and genetic (BFM protocol) parameters (**High risk**: p**=0.00000; Medium risk, Standard risk**: p**=0.0066)**. The main reason for the lack of further detectable prognostic effect of genetic subgrouping was given by correlation analysis: there was strong correla-

tion between hyperdiploid B, normal, TEL/AML1-positive subgroups and favourable clinical factors like age 2–6 yrs, low WBC and prae-B immunphenotype, likewise for pseudodiploid, tetraploid aberrations and unfavourable factors (infant/above 10 yrs of age, high WBC, CD-10 negativity/T immunphenotype). We performed multivariate analysis separately in nonT-nonB ALL cases and detected a significant negative prognostic effect for Philadelphia-positivity, tetraploidy and 11q aberrations respectively.

Discussion

A nationwide study was started in 1993 with the aim of providing genetic diagnosis to all newly diagnosed childhood ALL cases in Hungary. This seemed to be an achievable goal considering the yearly expected number of ALL children in Hungary (about 50–70/year) and that all of them are treated in one of the centers of the Hungarian Paediatric Oncology Network following unified diagnostic and therapeutic principles. Parallel application of chromosome analysis with flow cytometric DNA-index determination was suggested, completed by centromere-specific FISH and molecular genetic tests for the direct detection of specific translocations (ABL/BCR, MLL rearrangements, TEL/AML1, if possible MRD). All centres of the Paediatric Oncology Network and genetic laboratories were involved in the collaboration. During this ten-year study genetic diagnostic methods found widespread use and the results in childhood ALL in Hungary have improved a lot. While in 1993–4 as many as 48% of cases remained in the unknown subgroup, only 5% of those diagnosed in 2001–2.

DNA-index determination proved to be a basic, simple method to detect gross numerical changes (hyperdiploid A-B, hypodiploid, tetraploid) and different blast subpopulations. Application of flow cytometry at the end of the study was above 70%, and we still have to increase it. Although proportion and success rate of *cytogenetic analysis* increased (by 2002 nearing 100%), the rate of cases with aberrations remained low (average: 39% vs expected: 80–90%), due to technical problems like few and poor quality of metaphases, consequently failure of recognition of subtle changes [2, 6, 9, 10, 13, 19, 20, 24, 26, 32]. Although chromosome analysis proved to be the most informative method showing all numerical and structural aberrations, a detailed description of supernumerary chromosomes could be given in less than half of hyperdiploid B cases necessitating application of further methods. Addition of *FISH* provided identification of gains and losses of individual chromosomes, especially when karyotyping failed; revealed subpopulations of blasts, clarified some contradictive results [1, 2, 4, 11, 27]. *FISH* and *RT-PCR* offer a good opportunity to detect TEL/AML1 rearrangement, which is hidden for routine karyotyping [3, 7, 14]. The advantage of the FISH method is to make possible for geneticists to detect further additional aberrations of prognostic significance such as TEL deletion and extra AML1 signal. Other specific translocations (MLL, ABL/BCR) can also be screened by these methods using locus specific probes with a high success rate. Although most of specific translocations were detected by routine cytogenetic examination, 6 of 14 ABL/BCR positive patients were identified by molecular testing only (unsuccessful karyotyping). During the 10 years of our study the availability of molecular tests increased steeply becoming essential part of genetic diagnosis and basis for the detection of minimal residual disease [3, 7, 13].

It was rare when all three methods (cytogenetic examination, DNA-index, FISH) were available and concordant (mostly in hyperdiploid B cases) but the combination of the above methods often helped us make a correct genetic subgrouping even in the lack of succesful karyotyping or in cases with more than one cell lines. In cases of trisomies or tetrasomies of the smallest chromosomes DNA-index was often lower than 1.16 indicating that flow cytometry alone is not reliable even for the detection of hyperdiploid B patients. Comparing the spectrum of supernumerary chromosomes in hyperdiploid B cases revealed by cytogenetic test and FISH a slight difference was found: by routine karyotyping trisomies of chromosomes 4, 6, 8, 9, 10, 14, 17, 18, 19, 21, by FISH the involvement of 4, 6, 10, 12, 18, 21, and X chromosomes were detected most commonly. The difference can be explained by the use of different selected FISH probes and/or technical difficulties of chromosome analysis: misevaluation of extra chromosomes due to poor spreading of highly hyperdiploid metaphases [2, 4, 27].

As a result of the low rate of aberrant karyotypes due to technical problems the proportion of certain genetic subgroups (hyperdiploid B, pseudodiploid cases with specific translocations, TEL/AML1-positive, tetraploid) was lower then expected

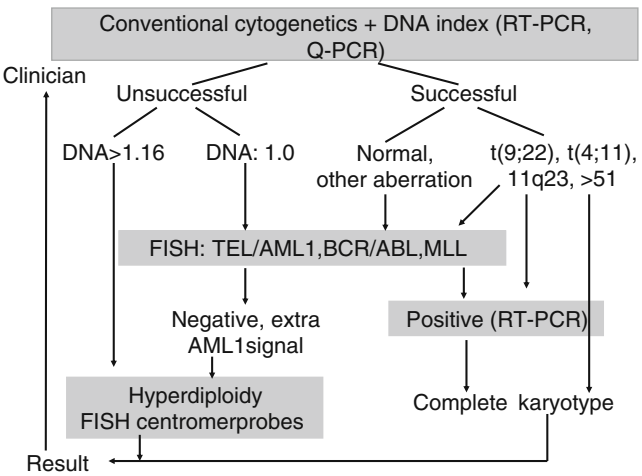


Fig. 8 Schedule of initial genetic tests in childhood ALL recommended for the future

[2, 6, 9, 13, 24]. The introduction and widespread use of flow cytometry and molecular genetic tests resulted in improvement by the second half of the study. The proportion of hyperdiploid B cases in B-cell precursor ALL was 26% in 2001–2 (versus 10% in 1993–4), that of pseudodiploid cases counted 6% (6%). This underlies the need for further improvement in traditional karyotyping which may reveal all types of numerical and structural aberrations. This is important because FISH or PCR can identify only those specific genetic alterations that are already suspected. The normal, TEL/AML1-positive subgroup was not detectable in 1993–4 due to the lack of the method but it's detection showed a fast increase from the mid-nineties up to 17% in 2001–2. The overall proportion of aberrations with a good prognosis was lower than expected: hyperdiploid B 18% (73/400), but nearing literature range by 2001–2 (25–30%). The normal, TEL/AML1-positive subgroup 9 % (36/400, literature: 15–20%); for the two favorable genetic subgroups together (109/307 successfully tested cases: 35.5%) [2, 6, 9, 13, 24]. Although one reason for this can be geographical heterogeneity in the distribution of aberrations but lack of DNA-index in 25% and TEL/AML1 RT-PCR/FISH examination in 30% of cases by 2002 may have contributed to the underdetection of these changes with good prognosis even in 2002 [19].

Prognosis of genetic subgroups differs significantly from each other: the cytogenetically normal, TEL/AML1 positive and hyperdiploid B cases proved to have the best, while pseudodiploid (including Ph+, 11q and other structural aberrations, deletions), and tetraploid cases the worst prognosis. Multivariate analysis identified congenital abnormalities, infancy, high initial WBC, delay/refuse in therapy, poor prednisone response and specific translocations (Ph, 11q) as main negative prognostic factors.

The conclusion of our experience in the 10 years' study is as follows:

- The combination of cytogenetic analysis, flow cytometric DNA-index determination completed by FISH, RT-PCR and in some cases by multicolour-FISH proved to be essential for proper genetic diagnosis and classification of patients into different genetic subgroups. Taking into account the advantages and disadvantages of all methods it is obvious that they have to be applied as complementary tests. The results of all genetic tests should be synthesized in the genetic subgrouping of patients.
- Specific genetic alterations (high hyperdiploidy, TEL/AML1 rearrangement, ABL/BCR and 11q aberrations) and genetic subgrouping proved to be of prognostic value. The present genetic subgroups are heterogeneous, therefore we have to make efforts to distinguish as many subgroups/prognostic modulators as possible.
- Prognostic value of certain genetic subgroups of ALL patients should be considered when choosing the most efficacious therapy: possible incorporation into international risk stratification and therapeutic protocols should be studied.
- For the future we recommend genetic laboratories to follow a new algorythm including parallel, complementary use of DNA-index, chromosome examination and molecular tests completed in special cases by FISH for the detection of specific gains/losses of chromosomes, translocations, complex aberrations and marker chromosomes (to detect cryptic aberrations and identify marker chromosomes multicolour-FISH, centralized in one laboratory in the country) [3, 9, 13, 14, 26] (Fig. 8).
- Coordination of methods, centralization, exchange of study samples are the best way to make the genetic diagnostic tests most reliable and available for all the patients and practitioners. This would encourage all laboratories to improve efficacy and reliability of cytogenetic analysis.

Although genetic alterations represent the underlying pathology in leukaemia, and determine disease characteristics and clinical course, their prognostic role acts through more trivial and easily detectable clinical parameters like age, initial WBC, immunphenotype [3, 8, 14, 32, 34]. These factors are summarized by standard risk stratification criteria. However, pathogenetically determined clinical entities show close correlation with commonly used prognostic factors and are also associated with distinct drug resistance profiles, which helps pediatric oncologists to make the best choice of therapy [14, 23, 34]. In order to further increase the number of survivals together with avoidance of toxicity of treatment, instead of more agressive chemotherapy, we have to make efforts to develop individualized, genotype-specific ie. risk-specific therapeutic schedules [5, 6, 9, 15–18, 23, 29, 30, 32]. Significant prognostic differences between various genetic subgroups call our attention to the importance of considering these genetic features in the development of future therapeutic agents and protocols [6, 9, 13, 23, 24, 29, 34]. Shedding light on underlying genetic aberrations in leukaemia is important not only for our understanding of etiology but also for the development of new drug combinations or genotype-specific blocking agents [3, 7, 8, 14–18, 28, 34].

We are aware of the fact that the initial period of regular genetic studies in ALL patients in Hungary has several shortcomings, out of which the most important is the lack of a standardized investigation system during the years in question. Various genetic methods were introduced gradually into routine testing, therefore the number of uniform, parallel investigations is rather low. Despite this, the results of the first 10-year period serve as a basis for comparison with those obtained in the recent 6-year period. Genetic studies carried out between 2003–

2008 are now being critically evaluated to produce more comparable results.

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