Ph.D. Thesis

High hyperdiploid acute lymphoblastic leukemia in childhood

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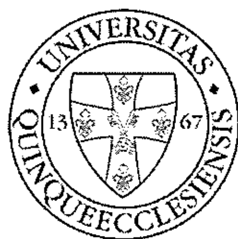

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Abbreviations

ALL	acute lymphoblastic leukemia
B-ALL	B-cell ALL
BFM	Berlin-Frankfurt-Münster
CCA	conventional cytogenetic analysis
CEP	centromer enumeration probe
CNA	copy number alteration
DAPI	4′,6-diamidine-2-phenyl indole
DI	DNA-index
DNA	deoxyribonucleic acid
EFS	event free survival
FISH	fluorescent in situ hibridisation
HeH	high hyperdiploid
HR	high risk
I-BFM	international BFM
iFISH	interphase fluorescent in situ hibridisation
iMN_8	modal chromosome number according to 8-parameter iFISH examination
IR	intermediete risk
LSI	locus specific identifier
MN	modal number
MRD	minimal residual disease
OS	overall survival
pEFS	probability of event free survival
pOS	probability of overall survival
PTE-KK	University of Pécs, Medical School, Department of Pathology
SNP	single nucleotide polymorphism
SR	standard risk
WHO	World Health Organization

1. Introduction

Acute lymphoblastic leukemia (ALL) is the most common malignancy in childhood. 60-70 new patients are diagnosed annually in Hungary. Though cure rate is around 85-90% with current treatment schedules in the developed countries, it still represents one of the most frequent cause of death due to malignancy in the age group of 1-18 years. When treating patients with ALL our goal is not only to improve their survival but also to decrease the short and long term toxicities of the treatment. In my thesis I have investigated the clinical and cytogenetic prognostic factors of high hyperdipoid acute lymphoblastic leukemia (HeH ALL), the largest genetic subgroup of childhood ALL, constituting 20-25% of all patients.

Cytogenetically HeH ALL is characterised by a non-random pattern of chromosomal gains with chromosome numbers of 51-65 according to the WHO 2016 classification of acute leukemias. The most frequent modal number is 55. The gains may involve all chromosomes, most frequently chromosome 4, 6, 10, 14, 17, 18, 21 and X, rarely 5 and 8. Chromosomal gains mainly result in formation of trisomies, sometimes tetrasomies and X-chromosome disomy for males. Pentasomy usually only affects chromosome 21.

Clinically HeH ALL is associated with young age (peaking at 2-4 years), low blood cell counts and precursor-B phenotype. These are characterstic features of favourable outcome and thus majority of patients receive standard intensity chemotherapy. End of induction remission rate is over 95% and overall survival (OS) exceeds 90%, recent studies reported on rates that exceed 95%. Despite these excellent results 20% of patients suffer a relaps, sometimes even years after completion of therapy. Event-free survival (EFS) is lower, 70-75% and 80-89% in recent larger series of children with HeH ALL. Since high hyperdiploid is the largest cytogenetic subgroup of childhood ALL this group accounts for the overall largest relaps proportion.

Thus HeH ALL is prognostically a heterogenous disease. While most of the patients can be cured with low intensity therapy, 20% of children relaps and eventually some of them succumb to the disease. Several investigations have been conducted in the past and are ongoing nowdays to identify those clinical and cytogenetic factors already at the time of diagnosis that are associated with poor outcome. Intensification of first-line therapy in these cases might result in higher chances for permanent remission. High modal chromosomal number and the presence of individual trisomies/ tetrasomies are associated with favourable outcome however the results of the different studies are contraversial.

2. Aims

1. Our aim was to analyse the copy number alterations of the eight most commonly involved chromosomes (4, 6, 10, 14, 17, 18, 21, and X) in HeH ALL on untreated bone marow samples of patients diagnosed with childhood precursor-B ALL at Medical University of Pécs, Pathology Institute. The 2 × 4 color consecutive iFISH assay with the relocation feature allowed us to obtain an eight-parameter correlated database at the single cell level. To the best of our knowledge such analyses had not been performed previously in pediatric acute lymphoblastic leukemia.
2. Patients were classified as high hyperdiploid according to the modal numbers identified by interphase FISH on eight chromosomes (iMN8). We established a database and collected the relevant prognostic clinical features and follow up data of the HeH patients out of this cohort from the Hungarian National Tumor Register and also from patients' medical records.
3. We investigated the prognostic significance of various clinical features and also the individual chromosomal gains and chromosomal pattern by univariate and multivariate analyses to reveal possible explanation for the heterogenous outcome of HeH ALL.
4. The aquisition of data correlated for 8-parameters revealed significant inter- and intra-patient heterogeneities of the chromosomal statuses in our cohort that had not been prevoiously described by studies based on the traditional diagnostic methodology, conventional cytogenetic analysis (CCA). Our aim was to analyse the characteristics of the observed clonal heterogeneity and it's potential prognostic effect. We also aimed to investigate the hierarchy of chromosomal gains so we could draw conclusions about the pathomechanism used for the gain of chromosomes in HeH ALL.

3. Matherials and methods

3.1. Patients

In total, 214 untreated bone marrow cell samples of children (1-18 years) with precursor-B ALL were investigated. Samples were archivated at -20°C, in 70% etanol. All patients were tested for t(9;22)(q34;q11.2) (*BCR-ABL1* gene fusion), t(v;11q23) (*KMT2A* rearrangement), t(12;21)(p13;q22) (*ETV6-RUNX1* gene fusion), t(1;19)(q23;p13.3) (*TCF3-PBX1* gene fusion) as well as Down syndrome and only patients negative for these alterations were included. Patients were treated in Hungarian pediatric onco-haematology centres with a central review of all diagnostic bone marrow samples at Medical University of Pécs, Pathology Institute. All patients received treatment according to either ALL BFM 1995 or ALL IC BFM 2002 protocol. Analysis of the relevant prognostic clinical features and follow up data was performed only for those patients identified as high hyperdiploid in this study. Our database included the following parameters: treating centre, date of birth, gender, date of diagnosis, presence of meningeal involvement, immunophenotype, white blood cell count at diagnosis, proportion of lymphoblasts in peripheral blood, treatment protocol, response to prednisone prephase, remission status on day 33, risk group, outcome of disease, in case of a relaps the date and site of relaps, type of therapy following relaps, date of last contact, in case of death date and cause of death. The study was approved by the Hungarian National Ethics Committee and conducted according to the Declaration of Helsinki. The work was supported by a Baross Gábor Project grant (OMFB-00342/2010) from the Hungarian National Development Agency.

3.2. Methods

3.2.1. iFISH probes

FISH utilizes fluorescently labeled DNA probes to chromosomal centromeres or unique loci to detect cells with numerical abnormalities. Alpha satellite DNA is based on a tandem, highly diverged repeat unit and localizes to the centromere of each human chromosome. Chromosome specific satellite DNA can be identified by cloned DNA probes specific for each chromosome. Centromere enumeration probes (CEP) for chromosomes 4, 6, 10, 17, 18, and X were available from our own laboratory. For chromosomes 14 and 21, locus specific identifiers (LSI) were designed to avoid cross-hybridisation (between chromosomes 14/22 and 13/21). The sensitivity, specificity and signal quality of the CEP and LSI probes were

analyzed on metaphase preparations in accordance with concerted guidelines. Two sets of probes were used, the first set (CEP set) contained CEP 4 (red), CEP 6 (green), CEP 10 (aqua), and CEP 18 (gold), and the second set (LSI&CEP) of probes were LSI 14 (red), LSI 21 (green), CEP 17 (aqua), and CEP X (gold) (Fig. 1).

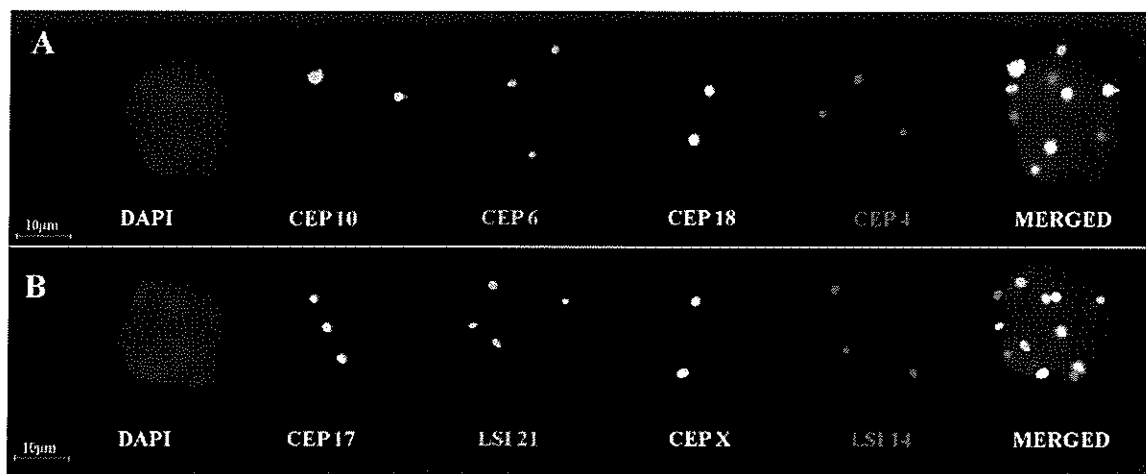

Figure 1. Consecutive 2x4 iFISH labeling of a target cell.
(A) First set of probes: CEP 4 (red), CEP 6 (green), CEP 10 (aqua), CEP 18 (gold); (B) rehybridization of the same target using the second set of probes: LSI 14 (red), LSI 21 (green), CEP 17 (aqua), CEP X (gold). Negative control nucleus, DAPI counterstain, 63X magnfiction.

3.2.2. Hibridisation

After preparation of leukemia samples fixed and stored in 70% ethanol three μl of the CEP hybridization mix (probes for chromosomes 4, 6, 10 and 18) was applied to each sample. Target and probe DNA were denatured together. Following hybridization posthybridization washing was performed then preparations were mounted with DAPI/Vectashield (Vysis, Downers Grove, IL), coverslipped and stored in the dark until analysis. For the second round of hybridization the coverslips were removed, the preparations were refixed, dehydrated then three μl of the LSI&CEP hybridization mix (probes for chromosomes 14, 17, 21, X) were applied. The denaturation and hybridization conditions were the same as in the first round.

3.2.3. Evaluation of the multiplex iFISH pattern

After each multiprobe labeling, slides were analyzed using a motorized multifunctional microscopic workstation. The microscope was equipped with a computer-controlled, motorized scanning stage, an X-Cyte metal halide lamp that transmitted light through quartz optical fibers, a motorized filter wheel and a cooled CCD camera. After hybridizing the first

set of four probes, slides were scanned using previously described fundamental nucleus recognition morphometric parameters, and images in the DAPI channel and the exact 3D coordinates of 1.000 single nuclei per sample were recorded. Signal patterns were evaluated manually by counting the iFISH dots on the recorded populations (3 signals: trisomy, 4 signals: tetrasomy, 5 signals: pentasomy) as seen in Figure 1. Analysis continued until clear-cut signal patterns in all four signal channels of at least 300 nuclei were recorded. After hybridizing the second set of FISH probes, slides were evaluated by using a semiautomated relocation of previously identified/evaluated cells, enabling investigation of eight-probe iFISH at the single cell level. In the course of data analysis only those cells with at least one evaluable signal for each of the eight probes were taken into consideration.

3.3. Statistical analysis

Univariate statistics were applied to reveal the possible differences between the subgroups according to various clinical (i.e.,gender, age, and WBC at onset) and cytogenetic (i.e., iMN8 and combinations of chromosomal gains) parameters. The pEFS and pOS were calculated using the Kaplan-Meier method and finally compared with a log rank test. Multivariate analysis using a Cox regression model was used to identify the factors with independent influences on the pEFS/pOS using IBM SPSS Statistics Version 20.0 (IBM Corp., Armonk, NY, USA). Statistical significance was taken as $p< 0.05$ in all analyses. For the mathematical modelling of clonal evolution, cluster and network analysis softwares, MEGA 5 and NETWORK 4.6.1. were used by the biostatistics of our working group.

4. Results

4.1. Identification of high hyperdiploid leukemias with iFISH

The 8-parameter correlated data acquisition allowed us to investigate the pattern of chromosomal gains at the single cell level. We observed a marked inter- and intra-patient heterogeneity of the chromosomal statuses in our cohort. Two types of subclones were indentified in the leukemia samples. Firstly, subclones by chromosome number implied all the cells with the same total gain of chromosomes, secondly unique subclones represented collections of cells with the very same constellation of the eight chromosomes, both according to the 2x4 iFISH assay. First, the number of subclones according to increments in chromosome number per patient were analyzed. For each leukemia samples, we obtained the frequency distribution of the subclones that had various chromosome numbers and the modal number (iMN8), which was defined as the chromosome number of the largest clone. According to this parameter, 89 out of 214 leukemia samples were hyperdiploid (iMN8 $\geq$ 47) and 48 were high hyperdiploid ($iMN_8 \geq 51$). The most frequent modal number in the HeH ALL group was 55.

4.2. Clinical characteristics of the HeH cohort

More than two-thirds of the 48 high hyperdiploid patients belonged to the age group of 1-6 years, 14 patients were older than 6 years and only 4 of 48 patients were older than 10 years. Median age of the whole cohort was 5.48 years. There were more females than males (28 vs. 20). More than half of the patients (25/ 48) were assigned to standard risk group. High initial white blood cell count (>20 G/l) that is considered to confer a less favourable prognosis was present in 13/ 48 patients, a count >50 G/l was observed only in five patients. Response to prednisone treatment was favourable in all but two patients, and on day 33 complete remission was confirmed in all patients except those who failed to reach this time point due to fulminant infection in induction. Thus the majority of our patints with high hyperdiploid ALL had favourable prognostic features according to the literature.

4.3. Prognostic relevance of clinical factors and chromosomal gains

The impact of clinical and cytogenetic factors on survival was also analysed. Based on pOS, using univariate analysis, the best separation of the subgroups by chromosomal gains could be obtained according to the iMN8 51–54 and 55–56 classifications of the cases. Univariate analysis revealed that only diagnostic white blood cell count proved to be significantly related

to the pEFS (p=0.019), and gender, age and iMN8 were not related. In contrast, only the iMN8 significantly separated the two groups (p = 0.046) according to the pOS. With respect to the gain of the individual chromosomes, chromosome 4 most significantly separated the subgroups (p = 0.006 for pEFS and p = 0.002 for pOS). The combined gains of the various chromosomes that also exhibited significant differences in pEFS, pOS or both were the following: 4-6, 4-10, 4-17, 4-18, 4-10-17 and 4-10-18. All of the leukemias with +4 also exhibited gains of chromosome 6, but the opposite relationship was not present.

Multivariate analysis with gender, initial white blood cell count and iMN8 as categorical variables and age as a continuous variable indicated that in addition to a lower white blood cell count (p=0.002), younger age (p=0.005) and a high iMN8 (p=0.008) also contributed significantly to superior pEFS values, but female gender did not (p=0.068). The same analysis of these four parameters revealed that in addition to the high iMN8 (p=0.004), younger age (p=0.003), a lower white blood cell count (p=0.015) and female gender (p=0.021) were also associated with superior pOS values. The combination of these four parameters with various chromosomal gains revealed that none of the chromosomal data exerted significant additional effects on the superior pEFS. In contrast, only individual gains of chromosome 4 were significantly associated (p=0.009) with a superior pOS.

4.4. Clonal heterogeneity

The 8-parameter correlated acquisition of the chromosomal gains allowed us to identify subclones not only by *chromosome number* but also according to the *unique karyotypes* detected in at least two copies per patient. Analyses based on the *subclones by chromosome number* revealed that the dominant subclones made up only an average of 34% of tumor cells and the average number of subclones was 9.9 in patients' samples. The frequency distribution of subclones of various leukemia samples exhibited various patterns, most frequently bell-shaped distributions were present in both the iMN8 51–54 and iMN8 55–56 groups, but with a more narrow base in the latter group of leukemias suggestive of less heterogeneity (Figure 2).

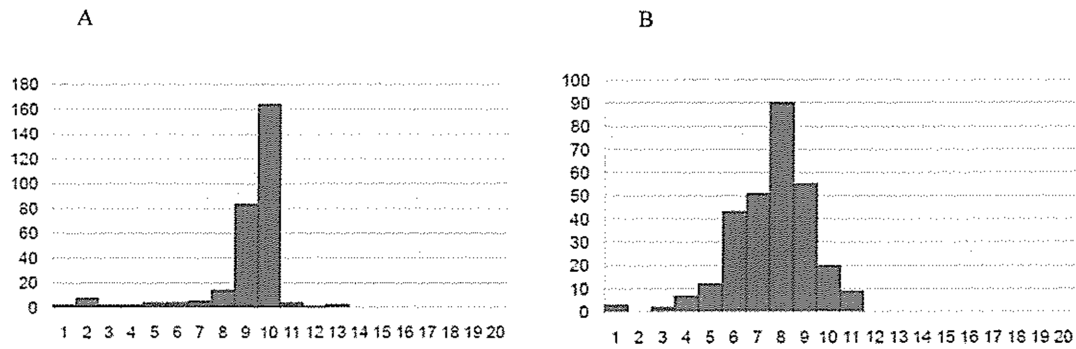

Figure 2. Frequency distributions of subclones according to the net gains of chromosomes, demonstarted in the case of two patines.

A: narrow based bell-shaped distribution in case of an iMN8 56 leukemia B: broad based bell-shaped distribution in case of an iMN8 54 leukemia. Y-axis: the number of cells analysed, X-axis: subclones according to net gains of chromosomes.

The clones by *unique karyotypes* were also analysed. After detecting the copy numbers of each of the eight chromosomes, a unique number of eight characters was set for each examined cell. Cells with the same unique karyotypes formed a unique kariotype subclone. One subclone by chromosome number might have been built up by more than one unique karyotype subclone. The pattern of unique karyotype subclones exhibited considerable heterogeneity. In some patients' samples dominant subclones were composed of several different unique kariotype subclones, while in other patients' samples only a few. The average number of unique subclones was significantly higher ($p = 0.028$) in the iMN8 51–54 group (29.9 ± 11.8) than in the patients in the iMN8 55–56 group (23.0 ± 9.2). Network analysis revealed a less complex pattern in the iMN8 55–56 group (Figure 3).

To clarify if chromosomes in individual leukemia samples have any specific order of acquisition, cluster analysis using unique subclones was performed. The clusters of chromosomal gains in individual patients were very diverse, with chromosome 21 and/or the X always being at the bases of the trees. The summarized clusters of all patients with iMN8 51-54 and iMN8 55-56 leukemia had highly consistent patterns using every algorithm (MEGA 5—NJ, UPGMA, and ME). In the iMN8 51–54 group, chromosomes 21, X, and 14 formed the base of the tree, and chromosomes 10 and 18 clustered together. Additionally, chromosomes 4 and 6 formed another cluster, whereas chromosome 17 did not consistently belong to the latter two groups. In the iMN8 55–56 group, the cluster formed by 10 and 18 was not present, but the topology was the same.

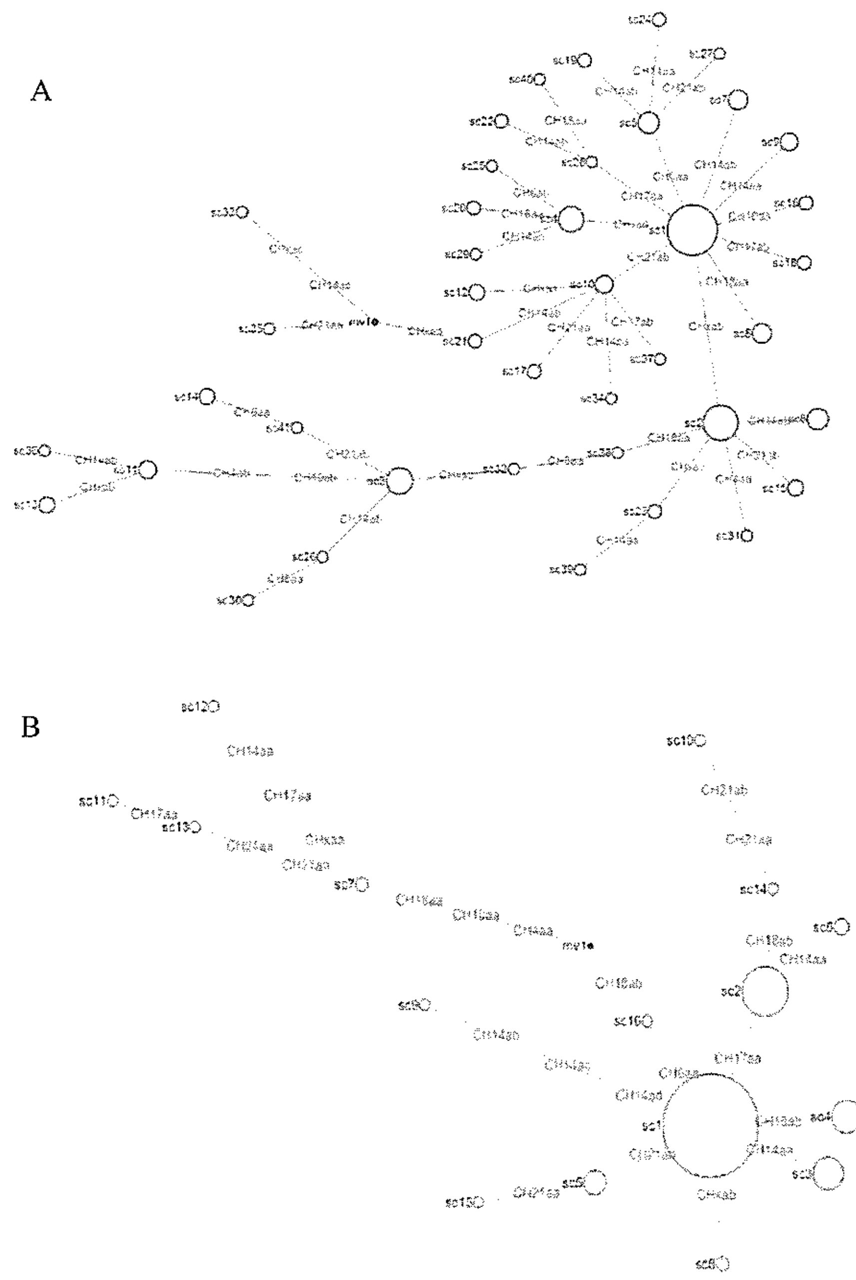

Figure 3. Network analysis of unique subclones in case of two patients.

A: a complex pattern (a patient with iMN8 54 leukemia) B: less complex, simple pattern (a patient with iMN8 54 leukemia). The yellow circles represent the unique subclones and the diameters are proportional to the number of cells with a given chromosome constellation. The lines connect the related subclones with indications of the chromosomes affected. The red dots are median vectors representing subclones that were not present or found.

5. Discussion

Current treatment protocols for childhood acute lymphoblastic leukemia incoporate several clinical and genetic parameters to identify children with different risk for relapse and treatment failure. Besides initial white blood cell count, age at diagnosis and response to treatment the gains of certain chromosomes and chromosome-combinations are also determining factors in the alignment to standard risk for some working groups. Most studies have detected specific effects of +4, +10, +17, +18 and range of MN values in univariate analyses, while the conclusions drawn from multivariate analyses have differed. The more recent studies are also controversial.

The reason for these controversial data is unknown, but besides the differences in the treatment protocols the role of the diagnostic methodology can not be excluded. The gold standard for detection of hyperdiploidity in ALL is conventional cytogenetic analysis and so in most of the publications either CCA or flow cytometry was performed. HeH ALL is known to have a lower plating efficiency and to often lack consistent abnormal clones thus, CCA, which in principal provides genome-wide data, in specific practice, e.g., in HeH ALL, might suffer from a significant loss of single-cell based correlated genomic data. Analysis of whole DNA content of non-dividing cells by flow cytometry does not inform us on what specific chromosomes were responsible for the detected net gains/ losses. On the contrary, when single-cell specific correlated 8-parameter iFISH data acquisition is applied using approximately fifteen-fold greater number of cells than usually targeted during CCA this technique might provide new information regarding the interrelations of chromosomal gains and their clinical correlations in HeH ALL.

In our present iFISH study we observed a marked heterogeneity in the subclonal architecture of high hyperdiploid leukemias. Two types of subclones were indentified in the leukemia samples. Firstly, subclones by chromosome number implied all the cells with the same total gain of chromosomes, secondly unique subclones represented collections of cells with the very same constellation of the eight chromosomes, both according to the 2x4 iFISH assay. Analyses based on the subclones by chromosome number revealed that the dominant subclones made up only an average of 34% of tumor cells and the average number of subclones was 9.9 in patients' samples. These data support an iFISH study of four chromosomes in a few precursor-B ALL patients that were not genetically subtyped (Talamo et al.), but are in contrast to the largest available CCA database revealing only one clone in

84% of ALL cases (Mitelman et al). The discrepancy between CCA and iFISH data could be explained by different growth advantages of the putative subclones resulting in clonal selection in case of CCA procedure as well as by the much higher representation of the targeted iFISH study. These might be the reasons that the new technologies seem to challenge the quasi monoclonal nature of childhood ALL according to CCA.

Subclonal and cellular heterogeneity observed in different solid tumors have been more frequently studied in ALL recently. Genetic heterogeneity may have important clinical implications, as subpopulations with distinct mutations may promote tumor adaptation to changes in environment (eg. chemotherapy) and they may also provide explanation for treatment failure, resistance, or relapse. In case of aneuploid malignancies aneuploidy can be either stable (eg. propagating abnormal clone that arises as a result of chromosomal segregational defect) or it can arise as a consequence of chromosomal instability characterised by an increased rate of copy number alterations, unstable kariotypes, cell-to-cell variability and complex subclonal architecture. This latter phenomenon had been recognised in HeH ALL prevoiusly (Blandin and Betts et al.), we also observed in our study but further investigations are necessary to clarify it's characteristics and prognostic significance.

In this study, among the gender, age, iMN8and WBC variables, only the latter proved to be a significant, and in conjunction with the cytogenetic data, this was an independent parameter in association with beneficial pEFS. The follow up of the clinical data indicated that the best separation and the only significant feature according to the overall survival analysis proved to be the iMN8 grouping of 51–54 (pOS:75%) vs. 55–56 (pOS:95.2%), according to the rule of higher being better. This massive difference was associated with a significant difference in subclonal heterogeneity in the two groups. However, the iMN8 was not an independent parameter but was overridden by the gain of chromosome 4 in the multivariate analysis (5 year pEFS 86.8%, p=0.006 and 5 yerar pOS: 92.1%, p=0.002).

Tumor cell aggressiveness is a double-edged sword; higher levels increase the risk to the patient but also increase the probability of the suicide of tumor cells via necrosis. This cut-off value might represent a balance between the extremes, which is also strongly supported by the peak incidence of HeH ALL of MN and iMN8 55 according to CCA and our multiparameter correlated iFISH studies, respectively. Leukemic cells may grow more favorably at this state of genomic imbalance due to a gain of specific chromosomes but without increased resistance upon therapy. As demonstrated by the network analysis, this was associated with more straightforward leukemogenesis and less heterogeneous chromosome segregation error, which resulted in a more homogenous subclonal landscape. The latter

finding could be beneficial for the outcome according to the not fully objective but self-explanatory rule that fighting against a multifaceted enemy is more difficult than fighting against a more simplified foe. Thus, although chromosomal gain is one defining parameter, it is not the ultimate parameter according to the pOS. This is because the iMN8 is overridden by the gain of chromosome 4, which is the only independent factor among all of the factors including the cytogenetic parameters. This finding might imply that specific dosages of proliferation-promoting and proliferation-inhibiting genes with a net reduction of survival capacity are conferred by the extra chromosome 4.

The iMN8 stratification was not independent from the extra chromosome 4. Our cluster analysis indicated that chromosome 4 forms the smallest independent cluster and always ranks at the eighth position in the course of the multistep acquisition of chromosomes. Because chromosome 21 is already very frequently tetrasomic with increasing iMN8, this implies that it is +4 that most frequently follows the acquisition of eight other chromosomes in the subclonal evolution, which results in a total of 9 extra chromosomes and an iMN8 (MN) of 55, which is the most common genetic constellation among HeH ALL patients.

Our results underline the significance of the gain of a specific chromosome in determining the outcome of childhood HeH ALL and suggest that modal number is secondary due to the conserved acquisition of chromosomes in this disease.

6. Summary

1. We analysed the copy number alterations of the eight most commonly involved chromosomes in high hyperdiploid acute lymhoblastic leukemia on bone marow samples of patients diagnosed with childhood precursor- B ALL. With the 2×4 color consecutive iFISH assay we investigated at least 300 cells of each 214 patients and obtained an eight-parameter correlated database at single cell level. To the best of our knowledge such analysis had not been performed previously in pediatric acute lymphoblastic leukemia.
2. According to the modal numbers identified by interphase FISH (iMN8) 48 patients were classified as high hyperdiploid ($iMN_8 \geq 51$). We established a database including the relevant prognostic clinical characteristics and follow up data of the HeH patients.
3. We found prognostic significance of high modal chromosome number. Patients with the highest modal numbers (iMN_8 55–56) had significantly better survival. With respect to the gain of the individual chromosomes, chromosome 4 was significantly associated with a superior pOS. We showed that combined gains of the various chromosomes (4-6, 4-10, 4-17, 4-18, 4-10-17 and 4-10-18) also exhibited significant improvement in pEFS, pOS or both.
4. We observed marked heterogeneity in the subclonal architecture of high hyperdiploid leukemias. We proved that the dominant subclones made up only an average of 34% of tumor cells and the average number of subclones was 9.9 in patients' samples. We described that the frequency distribution of subclones of various leukemia samples exhibit various patterns, most frequently bell-shaped distributions in both the iMN8 51–54 and iMN8 55–56 groups, but with a more narrow base in the latter group of leukemias suggestive of less heterogeneity.

   We proved that the pattern of unique karyotype subclones also exhibit considerable heterogeneity. The average number of unique subclones was significantly lower in the iMN8 55–56 group. Network analysis revealed a more homogenous subclonal landscape in this subgroup associated with a more straightforward leukemogenesis and better survival.

   Using cluster analysis, we identified a hierarchical order of acquision of chromosomes. Network and cluster analyses indicated that chromosomal gains mainly took place sequentially, one by one.

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8. Publications related to the thesis

Original articles

Vojcek Á, Pajor L. A gyermekkori akut limfoblasztos leukémia kedvező prognosztikai alcsoportja: magasan hiperdiploid leukémia. Magy Onkol. 2018;62:214-21.

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