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PROGNOSTIC MOLECULAR MARKERS OF CHILDHOOD LEUKEMIA

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‘I do not wish by any means to infer that the disease in question is incurable;
I hope on the contrary that for it too remedies will at length be discovered.’
RudolphVirchow, 1858

To my wonderful children
Lovisa, Didrik and Max

ABSTRACT

During the last decades there has been a dramatic increase in survival rates for childhood leukemia, resulting today in an overall survival close to 90 % for children with acute lymphoblastic leukemia (ALL). This accomplishment is largely due to treatment protocols based on careful risk group assessment guiding the type and intensity of the anti-leukemic treatment. Relapse remains still the most important cause of treatment failure and the outcome is then much more dismal. The aim of this thesis was to increase the understanding how genetic aberrations and alterations in cell cycle regulating proteins in the leukemic cells, contribute to pathogenesis and prognosis of childhood ALL.

On behalf of the Nordic Society of Pediatric Hematology and Oncology (NOPHO), we investigated the frequency and clinical implications of the genetic aberration dic(9;20)(p13.2;q11.2), which easily escapes detection by chromosome banding analysis alone. We obtained material and performed interphase FISH on more than half (53%) of all Nordic pediatric patients diagnosed with B-cell precursor (BCP) ALL during 2001-2006. 4.6% (25/542) had dic(9;20), making it the third most common genetic subgroup of BCP ALL after high hyperdiploidy and t(12;21)(p13;q22). 5-year event-free survival and overall survival were 0.68 and 0.83, respectively, which were significantly worse than for t(12;21)-positive cases and for cases with high hyperdiploidy. We conclude that dic(9;20) is twice as common as previously surmised and that it is a non-standard risk abnormality, which should not receive standard risk therapy.

Neonatal dried blood spots (DBS), or Guthrie cards, have been used to demonstrate the prenatal origin of clonal leukemia-specific aberrations in several genetic subgroups of childhood ALL. In addition, the ETV6/RUNX1 fusion transcript has been detected in 1 % of normal cord blood samples. We report that transcripts encoding β-actin are readily detectable in RNA isolated from neonatal DBS stored for up to 20 years and that no significant loss of transcripts appeared to occur over time. The lack of significant decay of RNA in DBS stored for up to 20 years, suggests that such filters are useful for studies of RNA determinants of diseases with an onset in childhood or in adult life. We also investigated DBS from 14 children, who later were diagnosed with leukemia (8 with t(12;21) ALL and six with t(9;22) ALL or chronic myeloid leukemia) and 14 age-matched controls, for the presence of leukemia-specific fusion transcripts. Transcripts for ETV6/RUNX1 and BCR/ABL could not be detected by RT-PCR, using clone specific primers, in any of the samples.

PTEN and SHP1 are tumor suppressor genes involved in the regulation of cell cycle control and apoptosis. Disturbance in the expression and signaling pathways of VEGF has been linked to pathogenesis of hematological malignancies. We have investigated the expression of PTEN, SHP 1, VEGF and two of its receptors, VEGFR-1 and VEGFR-2, in childhood BCP ALL and non malignant controls by immunohistochemistry. PTEN was over expressed while SHP1 showed a low expression in diagnostic ALL samples, compared to non-malignant controls. ALL samples had significantly increased expression of VEGFR-1 compared to no expression in the non-malignant group, indicating a link between VEGFR-1 protein expression and pre-B ALL. Our sample size was small and the potential role of these proteins in diagnosis and prognosis of childhood ALL needs to be further investigated.

ORIGINAL ARTICLES

This thesis is based on the following articles, which will be referred to in the text by their Roman numerals as listed below:

I. Erik Forestier*, **Fredrika Gauffin***, Mette K. Andersen, Kirsi Autio, Georg Borgström, Irina Golovleva, Britt Gustafsson, Sverre Heim, Kristina Heinonen, Mats Heyman, Randi Hovland, Johann H. Johannsson, Gitte Kerndrup, Richard Rosenquist, Jacqueline Schoumans, Birgitta Swolin, Bertil Johansson, and Ann Nordgren on behalf of the Nordic Society of Pediatric Hematology and Oncology (NOPHO), the Swedish Cytogenetic Leukemia Study Group (SCLSG), and the NOPHO Leukemia Cytogenetic Study Group (NLCSG). (*Authors contributed equally.) Clinical and Cytogenetic Features of Pediatric dic(9;20)(p13.2;q11.2)-Positive B-Cell Precursor Acute Lymphoblastic Leukemias: A Nordic Series of 24 Cases and Review of the Literature. *Genes, Chromosomes & Cancer*. 2008, 47:149–158.

II. Vasilios Zachariadis, **Fredrika Gauffin**, Ekaterina Kuchinskaya, Mats Heyman, Jacqueline Schoumans, Elisabeth Blennow, Britt Gustafsson, Gisela Barbany, Irina Golovleva, Hans Ehrencrona, Lucia Cavalier Franco, Lars Palmqvist, Gudmar Lönnerholm, Magnus Nordenskjöld, Bertil Johansson, Erik Forestier, and Ann Nordgren, for the Nordic Society of Pediatric Hematology and Oncology (NOPHO) and the Swedish Cytogenetic Leukemia Study Group (SCLSG). The dic(9;20)(p13.2;q11.2) is an emerging non-standard risk aberration in childhood B-cell precursor acute lymphoblastic leukemia: results from the NOPHO ALL-2000 trial. *Manuscript.*

III. **Fredrika Gauffin**, Ann Nordgren, Gisela Barbany, Britt Gustafsson, and Håkan Karlsson. Quantitation of RNA decay in dried blood spots during 20 years of storage. *Clinical Chemistry and Laboratory Medicine*. 2009, 47(12):1467-1469.

IV. **Fredrika Gauffin**, Gisela Barbany, Håkan Karlsson, Mats Heyman, Ingrid Thörn, Magnus Nordenskjöld, Britt Gustafsson and Ann Nordgren. ETV6/RUNX1 and BCR/ABL are not found on RNA transcript level in neonatal dried blood spots from children with leukemia. *Manuscript.*

V. **Fredrika Gauffin**, Eva Diffner, Bertil Gustafsson, Ann Nordgren, Anette Gjörloff Wingren, Birgitta Sander, Jenny Liao Persson, and Britt Gustafsson. Expression of PTEN and SHP1, investigated from tissue micro arrays in pediatric acute lymphoblastic leukemia. *Pediatric Hematology and Oncology*. 2009, 26:48–56.

VI. Eva Diffner, **Fredrika Gauffin**, Lola Anagnostaki, Ann Nordgren, Bertil Gustafsson, Birgitta Sander, Britt Gustafsson, and Jenny Liao Persson. Expression of VEGF and VEGF receptors in childhood precursor B-cell acute lymphoblastic leukemia evaluated by immunohistochemistry. *Journal of Pediatric Hematolology/Oncology*. 2009 Sep;31(9):696-701.

CONTENTS

ABBREVIATIONS

ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
BCP	B-cell precursor
CDC	Cell division control
CGH	Comparative genomic hybridization
CML	Chronic myeloid leukemia
Ct	Threshold cycle
DBS	Dried blood spots
EFS	Event-free survival
FISH	Fluorescent *in situ* hybridization
HeH	High hyperdiploid
HPC	Hematopoietic progenitor cell
HSC	Hematopoietic stem cell
IgH	Immunoglobulin heavy chain
LOH	Loss of heterozygosity
MDS	Myelodysplastic syndrome
M-FISH	Multiplex fluorescent *in situ* hybridization
MRD	Minimal residual disease
mTOR	Mammalian target of rapamycin
NOPHO	Nordic Society of Pediatric Hematology and Oncology
OS	Overall survival
Ph	Philadelphia chromosome
PI3K	Phosphoinositide 3-kinase
PTEN	Phosphatase and tensin homologue
RT- PCR	Reverse transcriptase polymerase chain reaction
RQ-PCR	Real time quantitative polymerase chain reaction
SHP1	Src homology 2 domain-containing protein tyrosine phosphatase
SKY	Spectral karyotyping
TCR	T-cell receptor
TMA	Tissue micro arrray
VEGF	Vascular endothelial growth factor
WBC	White blood cell

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common malignancy in childhood, accounting for approximately 25% of all pediatric oncologic diseases. During the last decades there has been a dramatic increase in survival rates for childhood leukemia. In the 1950s, the mean survival time for children diagnosed with ALL was only 6 months, and the vast majority of the children died from their disease. Today almost 80 % of the children with ALL will have a long term event free survival (EFS) and the overall survival (OS) is close to 90 %.[1] This dramatic increase in survival is largely due to a careful risk group assessment with prognostic biomarkers guiding the intensity of the treatment protocol, combined with a better control of infections.

However, the therapy protocols used are very toxic for the patient and long term adverse side effects are not uncommon. The etiology of ALL remains in most cases unknown and there is thus no way of preventing the disease. Future treatment for leukemia will involve defining the molecular pathways underlying the pathogenesis of the disease and further investigation of the pharmacogenetic factors of the host.

History

Leukemia

The first recognition of leukemia is from October 1845 when the Scottish pathologist John Hughes Bennet published a detailed autopsy report of a patient, clinically described by the Edinburgh physician David Craige, entitled "Case of Hypertrophy of the Spleen and Liver in which Death took place from Suppuration of the Blood". 6 weeks later the German pathologist Rudolf Virchow described a disease, with an increased number of colorless cells in the blood, which he called "*weisses Blut*". Bennets drawings of the cells he saw in the microscope, affecting the whole blood mass, was published 1852 and were the first illustrations of the blood cells of a

patient with leukemia. Virchow later described the non-infectious nature of leukemia and divided the disease into a lymphocytic and a splenic type, the latter now called myeloid. In 1857 Nikolaus Friedreich, a pathologist in Wurzburg, recognized a case of acute leukemia of the lymphatic type with a very short time between presentation and death, which differed from the earlier described chronic forms. An important discovery came in 1868 when Ernst Neuman, professor of Pathological Anatomy at Konigsberg, suggested the bone marrow being the site for blood cells formation and the origin of the disease.[2]

Cytogenetics

Walther Flemming, a German biologist, published the first illustrations of human chromosomes in 1882 and the word 'chromosome' was introduced in 1888 by the German anatomist Heinrich von Waldeyer. Already at the end of the 19th century the German pathologist David Hansemann described the occurrence of nuclear and mitotic irregularities in cancer cells. Around 1900 the German scientist Theodor Boveri and the American scientist Water Sutton developed a theory that the determinants of inheritance were carried on the chromosomes, and in 1914 Boveri presented a somatic mutation theory of cancer in his book "*Zur frage der Entstehung maligner tumoren*". He suggested that cancer cells may have abnormal chromosomes, and that an unbalanced chromosome constitution could cause malignant tumors. In 1923 Painter, an American zoologist suggested the human genome having 48 chromosomes. It wasn't until 1956 the Dutch scientist Joe Tjio, working with the Swedish Professor Albert Levan at the University of Lund in Sweden, reported that the human diploid chromosome number appeared to be 46, not 48 which had been a dogma for over 30 years![3, 4]

In 1960 Peter Nowell introduced a mitogen agent making it possible to use peripheral blood samples, instead of bone marrow aspiration, for chromosome studies. The same year the discovery of the "Philadelphia chromosome" in chronic myeloid leukemia, the first described somatic primary chromosome aberration in man, demonstrated for the first time an association between cancer and chromosomes.[5] Not until the introduction of banding techniques in the 1970s, was it possible to identify and diagnose structural abnormalities in each chromosome. At this time a fluorescent staining technique was used, and and Torbjörn Caspersson, a Swedish cellbiologist

and geneticist, and his co-workers could describe a unique banding pattern for each human chromosome.[6] However, the fluorescence intensity quickly faded and for routine use in a clinical setting, the G-banding technique based on the application of trypsin followed by Giemsa staining became the most accepted method worldwide. This revolutionized the study of cancer cytogenetics and Dr Janet Rowley could in 1973, using the new staining techniques, describe the Philadelphia chromosome as a translocation between the long arm of chromosome 22 and the long arm of chromosome 9 (Figure 1). [7]

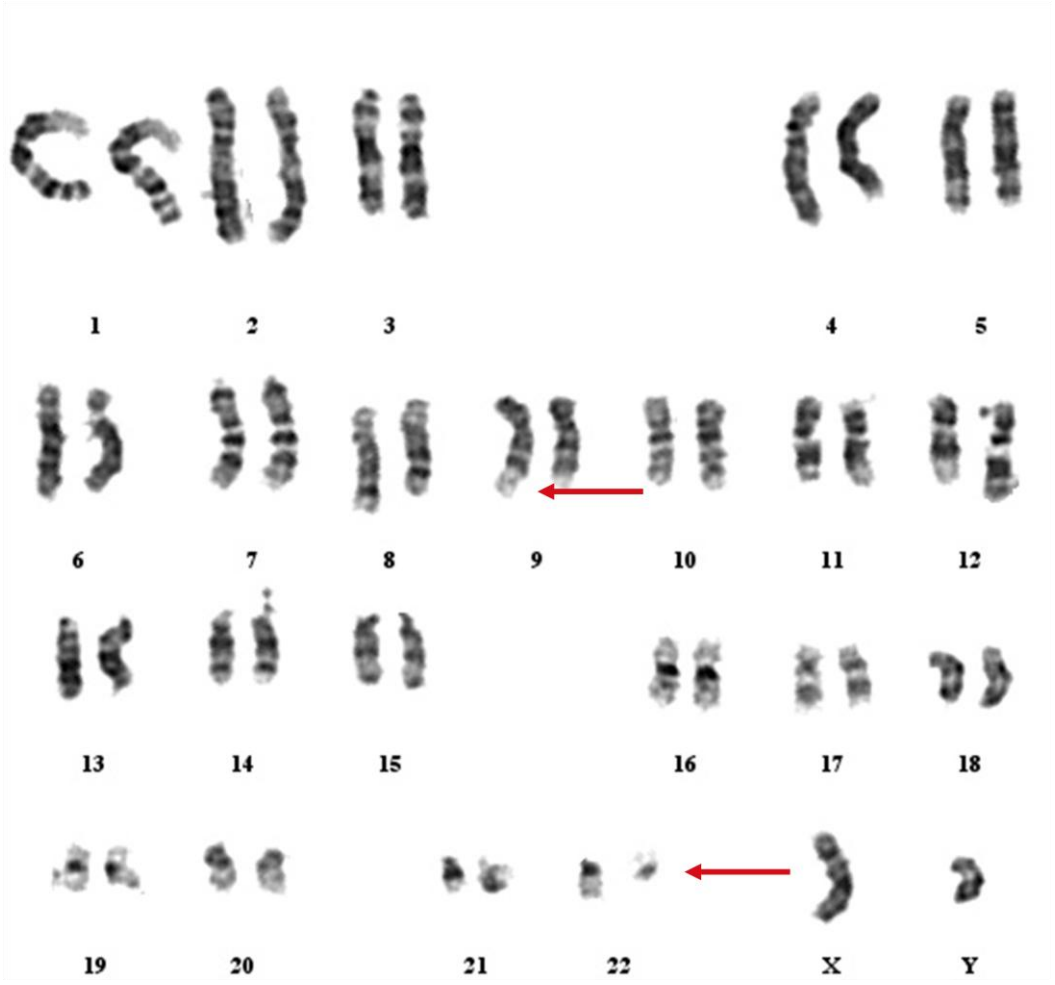

Figure 1. A translocation between the long arm of chromosome 22 and the long arm of chromosome 9 creates the Philadelphia chromosome indicated with an arrow at chromosome 22.

New techniques have since been developed which allow us to study the genetic material in more detail, combining traditional cytogenetic methods and modern molecular genetics, i.e. molecular cytogenetics. Fluorescent *in situ* hybridization (FISH) was first described in 1977,[8] and has become an important technique that bridges the methodological gap in resolution between chromosome banding and

molecular techniques. Comparative genomic hybridization (CGH), a technique that may be used to detect and map genomic imbalances, was first described in the early '90s by Anne Kallionemi et al.[9] In 1996 two new FISH methods were introduced, multiplex FISH (M-FISH) and spectral karyotyping (SKY), that allowed the simultaneous hybridisation and analysis of all human chromosomes in different colors.[10, 11]

Cell cycle regulation

Already in 1855 Virchow realized that cells only arise from pre-existing cells, which by the turn of the last century resulted in microscopists and embryologists describing the cytology of cell division in great detail, but they could only speculate about the underlying mechanisms.[12] The knowledge we have today of cell cycle molecular mechanisms and control has been brought to us through the joined forces of cell biologists, biochemists and geneticists in the late 1970s and 1980s. In the 1970s geneticists Paul Nurse and Leland Hartwell identified the first fission yeast CDC (cell division control) genes and explored the genetic control of the cell cycle [13, 14]. At the same time embryologists described chromosome condensation activity before initiating meiotic division in frog oocytes.[15] Biochemist Tim Hunt's research group studied sea urchins and discovered cyclins, proteins synthesized and destroyed in synchrony with cell cycle division.[16] Nurse and Hartwell together with Tim Hunt were awarded the Nobel Prize in 2001. With the development of recombinant DNA methodology in the late 1970s and '80s it was possible to clone, characterize, and manipulate cell cycle genes. Using relatively routine methodologies the knowledge of how the cell cycle is regulated in normal and cancer cells advanced rapidly.

The first oncogene was discovered in1970 in a chicken retrovirus and was named src by Steve Martin.[17] During the 70s and 80s several groups explored the relationship between oncogenes and chromosome abnormalities in human tumors.[18] Bishop and Varmus were awarded the Nobel Prize in 1989 for their research on how proto-oncogenes are transformed into oncogenes.[19]

Treatment of childhood leukemia

If untreated, childhood ALL is a fatal disease. In the late 1940s Farber used the folic acid antagonist aminopterin to induce temporary remission in childhood leukemia.

This was the beginning of modern treatment of leukemia with cytostatic drugs, and the closely related antifolate methotrexate is still a cornerstone in antileukemic therapy. The therapeutic value of corticosteroids was discovered in 1949 and by 1950 Hitchings and Elion used rational drug design to create 6-mercaptopurin, the first truly effective leukemia drug.[2] Many patients entered remission with these new drugs, but they later relapsed and died. In the 1960s and 1970s single drug therapy developed towards multidrug therapy schedules with the introduction of many new cytostatic drugs, improving the induction-remission rates as well as the duration of remission.[20]

CNS prophylaxis with radiation therapy and intrathecal injections of methotrexate was introduced in the 1970s and in the next decade re-induction therapy and use of higher doses of methotrexate was added.[20] With increasing intensity of the treatment, bone marrow toxicity was limiting. Very high doses of cytostatic drugs and radiation could be used to eliminate the leukemia in patients with highest risk of treatment failure, with the introduction of allogenic hematopoietic stem cell transplantation (HSCT) in the late 1970s.[21] In addition the graft versus leukemia effect was discovered.[22] During the last 3 decades no major therapeutic breakthrough has been made, except from imatinib mesylate and second-generation ABL-kinase inhibitors used to treat Philadelphia chromosome-positive ALL and chronic myeloid leukemia (CML).[23] In a hopefully near future the defining of the molecular pathogenesis of leukemia will provide us with new genes, whose protein products are suitable for targeted therapy.

The introduction of therapy protocols for childhood ALL based on risk group assessment has greatly contributed to the increased survival rates. Different prognostic markers at diagnosis – such as age, sex, clinical parameters (white blood count, mediastinal mass, CNS involvement), immunophenotype and karyotype of the leukemic lymphoblast – have placed the children into risk groups deciding the therapeutic intensity of the treatment.[24] Today remission rates in children exceed 95% and the overall event-free survival (EFS) is almost 80 %.[1]

The origin of childhood leukemia

ALL can occur in all ages but have in developed countries a characteristic peak around 2-5 years of age and in the Nordic countries an incidence of 5 cases per 100 000 and year.[25,26] Children diagnosed with ALL at an age less than 1 year are classified as infant ALL, depending of the unique biological characteristics of the disease in this age group. Several genetic syndromes, such as trisomy 21, Bloom syndrome, neurofibromatosis, Shwachman-Diamond syndrome and ataxia-telangiectasia, have been associated with an increased risk of developing childhood leukemia, but they account for only a small part (< 5 %) of all cases.[25, 27, 28] Incidence, age distribution and subtypes of childhood leukemia differ geographically and according to the socioeconomic status of the country. The highest incidence rates are reported from Costa Rica and Ecuador. Middle to high income countries all have a rather high incidence rate and the lowest reported incidence is from Malawi and Mali. Developing countries tend to have no childhood incidence peak.[29]

Tracing back to birth

The first report of leukemia in twins is from 1882.[30] Concordance rates for leukemia among monozygotic twins are extraordinarily high, suggesting there might be a genetic predisposition or the presence of potential leukemogenic factors of the shared intrauterine environment.[31] However, neither non-twin siblings nor dizygotic twins with separate placentas seem to have an elevated risk of leukemia. Identical genomic breakpoints in monozygotic twins concordant for infant leukaemia described by Ford et al in 1993,[32] rather suggested the initial leukemic clone is developed *in utero* in one twin and then transmitted to the other twin before birth.[30, 32, 33]

Neonatal dried blood spots (DBS), or Guthrie cards, have been used to demonstrate the prenatal origin of several types of childhood leukemia. Stored neonatal DBS from children who developed leukemia have been analyzed for chromosomal aberrations found at diagnosis, including t(4;11),[34] t(12;21),[33, 35, 36] t(8;21)[37] and hyperdiploidy.[38, 39] Genomic DNA from each leukemic patient was amplified by long distance PCR and the unique patient gene rearrangement was sequenced. A PCR assay was subsequently designed for the breakpoint junction to determine if the sequence was present in the DNA isolated from the DBS. In ALL cases lacking specific

chromosomal translocations, analysis of clone specific immunoglobulin heavy chain (IgH) and T-cell receptor (TCR) gene rearrangements have provided indirect support for a prenatal origin.[40-42] Preleukemic cells were found by analyzing DBS for clonotypic IgH in about 60 % of children with B-cell precursor (BCP) ALL, with the highest incidence of 90 % found in hyperdiploid leukemia.[43, 44]

In cord blood from healthy children mRNA representing the fusion genes for ETV6/RUNX1, t(12;21), or RUNX1-ETV, t(8;21), have been detected in about 1 % of the samples at cell levels of 10^{-3} – 10^{-4}.[45] This is 100-fold greater than the risk of developing the corresponding leukemia. In adult healthy blood donors the prevalence of ETV6/RUNX1 positive samples was about the same, but positive samples had much lower levels of ETV6/RUNX1 positive cells.[46] This is consistent with a two-hit model of the development of childhood ALL, in which the first event occurs *in utero* and the second required event occurs postnatally. Maia et al [47] describes a triplet set with 2 monozygotic twins with t(12;21) ALL. A clonotypic genomic ETV6/RUNX1 sequence was shared by the diagnostic leukemic cells of the twins and present in their neonatal blood spots. Associated chromosomal deletions were distinct for each twin, suggesting they were acquired as postnatal, secondary events independently. The same ETV6/RUNX1 sequence was amplifiable in the neonatal blood spot of the healthy dizygotic triplet, but not detectable in peripheral blood after 2 years. This suggests that there has been an entering of the preleukemic cells through the maternal circulation. Dizygotic twins, with separate placentas, do not seem to have an elevated risk of leukemia.

Support for this two-step model of leukemogenesis is found in animal models with the ETV6/RUNX1 fusion gene. Mice transfected with murine stem cells with ETV6/RUNX1-expressing vectors developed sustained expansion of B-cell precursors and a partial block of differentiation, but no overt leukemia.[48] Morrow et al[49] transduced murine fetal liver hematopoietic progenitor cells (HPC) with a retroviral vector expressing ETV6/RUNX1 fusion showing a promotion of B-lineage development *in vitro*. However, mice that received transplants of ETV6/RUNX1 HPCs did not develop leukemia, despite ETV6/RUNX1 HPCs showing competitive advantage in reconstituting B-cell and myeloid cell lineage. Taken together, these results provide direct evidence for preleukemic activity of ETV6/RUNX1 and confirm

previous experimental observations that ETV6/RUNX1 is necessary but insufficient for leukemogenesis.

First and second hit

Acute leukemia is a consequence of malignant transformation of a single HPC, by a sequence of molecular events disrupting the process of differentiation, limiting the proliferation that characterizes normal hematopoiesis, and generating a leukemic clone capable of expanding by indefinite self-renewal. A preleukemic stem cell clone can be generated by a first mutation *in utero* which, in a minority of children, progresses to leukemia after receiving further postnatal genetic hits. We know very little about what gives rise to the leukemic clone found at birth, and what events make this clone develop into the disease.

A genetic predisposition seems to be present in only a small proportion of childhood leukemia and is almost entirely accounted for by Down's syndrome.[25] The age peak between 2–5 years of age and higher incidence of the most common leukemia subtype in affluent societies, suggest a contributory role of socioeconomic factors.[50] Many environmental exposures have been suggested to cause leukemia in children including maternal consumption of dietary topoisomerase II inhibitors and biflavonids,[51, 52] or pre- and postnatal exposure of ionizing[53] and non-ionizing radiation[54]. Despite a multitude of epidemiological studies, the only established causal exposure for childhood leukemia is ionizing radiation.[53] The extent to which environmental factors contribute to leukemogenesis in children has not been clarified, and it is not likely that the majority of childhood leukemia cases are attributable to a single exclusive cause. Most likely, gene-environment interactions influence the risk of leukemia development.

An infection, possibly a virus, has long been regarded as a likely cause of childhood leukemia. However, molecular screening for candidate viruses has, to date, produced negative results.[55] Adenovirus DNA was detected by our group in 13 of 49 neonatal blood spots from ALL patients but only in 3 of 47 controls, suggesting a correlation between prenatal adenovirus infection and the development of ALL. However, a subsequent study of twice as many pediatric ALL patients in California failed to find adenovirus DNA in any of the blood spots,[56] and in a larger study from our group

with 243 children who later developed ALL and 486 matched controls Adenovirus DNA was reliably detected from only two subjects, both of whom developed ALL.[57] In conclusion, adenovirus in DBS is demonstrated only infrequently and the possible association between adenovirus and the development of leukemia remains unproven.

In 1988, two hypotheses were proposed regarding how infection could still be an important causal factor in childhood leukemia. The Kinlen hypothesis[58] emerged in the context of the observed leukemia clusters around nuclear reprocessing plants in Sellafield, Egland. Since the radiation exposure levels were too low to explain the increased leukemia incidence, Kinlen argued that the unusual population mixing caused by migrant workers recruited to these remote regions might be the causative factor. His hypothesis implies that childhood leukemia clusters might be an unusual outcome of a common but relatively nonpathological yet unidentified infection, which is transmitted preferentially during periods of population mixing. In the same year, Greaves[59] proposed an alternative but related delayed-infection hypothesis for childhood leukemia. He explained the observed correlation between lifestyle and the characteristic age peak among 2–5 years old children by an inadequate priming of the immune system. Children in more affluent 'hygienic' societies, are exposed to a narrow range of infections early in life, and thus remain immunologically more naive for a longer period of time. Malignant progression of a preleukemic clone is hypothesized to occur as part of an abnormal immune response to a common infectious agent during delayed exposure. The most conclusive epidemiological studies have been based on the day-care attendance in early life, as a parameter for early and broad exposure to common infections. The majority of these studies have indicated that there is a protective role for early institutional day care, but the evidence is not conclusive.[55, 60]

Chromosome abnormalities of childhood ALL

At the time of initial diagnosis of childhood ALL the morphology and immunophenotype of the leukemic clone are carefully assessed. Detailed immunophenotyping is necessary for subclassification according to the maturation stage of the B- and T-cell lineage lymphocytes, and as a baseline investigation for the

monitoring of minimal residual disease (MRD) to be able to measure treatment response. Analysis of the patients karyotype and response to treatment provides crucial information of prognostic importance, and allows for a more individualized approach to the choice of treatment strategy.

During the last decades many chromosomal abnormalities have been described in the leukemic clones of childhood ALL. Routine karyotyping at diagnosis identify genetic changes in 65-70 % of the pediatric ALL patients.[61, 62] When combining interphase FISH, using probes to detect the common ALL translocations, and array CGH aberrations were found in 90 % of children with a normal karyotype by G-banding.[63] The genetic changes can be divided into numerical and structural abnormalities, and some of these have been found to have prognostic impact and are used at diagnosis to decide the intensity of the treatment protocol. Common numerical abnormalities include hypodiploidy (< 45 chromosomes), associated with a high risk of treatment failure and a poor prognosis, and high hyperdiploidy (HeH) (52-60 chromosomes), associated with a good response to treatment and continuous remission (Figure 2).[1, 64, 65]

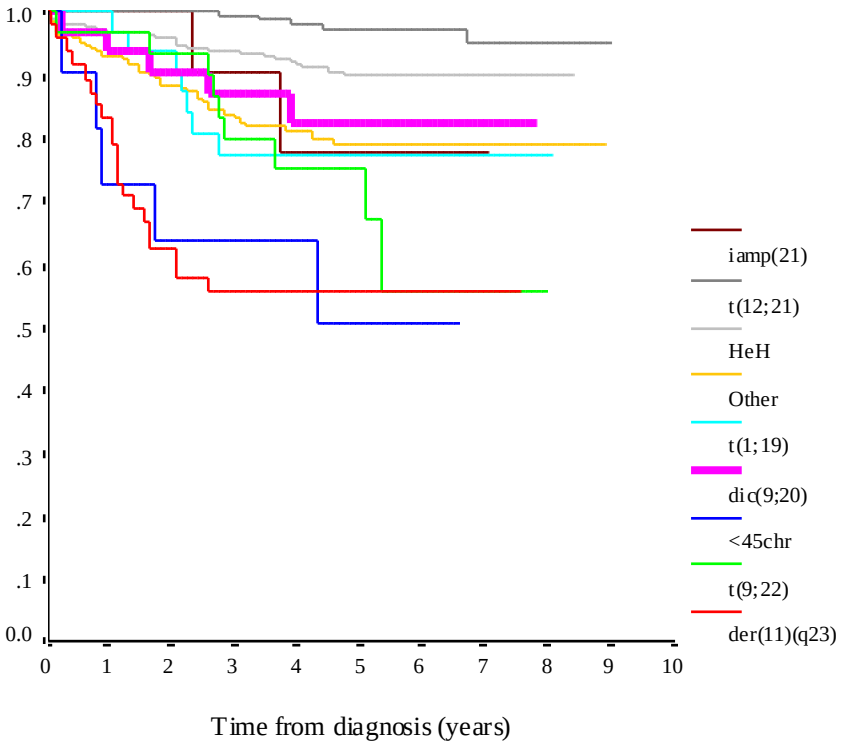

Figure 2. *The overall survival in different cytogenetic subgroups in the cohort of 542 BCP-ALLs included in article II.*

Many recurrent structural aberrations are translocations influencing the regulatory mechanisms of important cell regulating genes, such as growth factors, receptors and transcription factors.[66, 67] Sometimes the translocation results in a functional fusion gene, such as the t(12;21) resulting in the ETV6/RUNX1 fusion gene or t(9;22) resulting in the BCR/ABL fusion gene. The chromosomal changes might be primary changes, involved in the transformation of the preleukemic clone, or secondary changes promoting the progression of the preleukemic clone into overt leukemia.[68]

ETV6/RUNX1 rearrangement

The t(12;21)(p13;q22), resulting in ETV6/RUNX1 (former called TEL/AML1) gene rearrangement is the most frequent structural aberration in childhood ALL and is found in about 25 % of the cases.[69] The translocation fuses the genes ETV6 and RUNX1, coding for two different transcription factors, and the resulting protein seems to lead to hampered early lymphocyte development.[25] This aberration is not visible by conventional chromosomal analysis, and has to be detected by fluorescent *in situ* hybridization (FISH) or reverse transcriptase polymerase chain reaction (RT-PCR).[70]

Twin studies and analysis of neonatal blood spots have shown that ETV6/RUNX1 often arises prenatally as an early initiating mutation.[33, 35, 36] In addition to the t(12;21), more than 50-70 % of the patients display additional chromosome aberrations, mainly unbalanced abnormalities, with loss of 12p and gain of chromosome 21 as the most common.[69] The patients are usually in the childhood peak, and have no bad prognostic markers. t(12;21) is thus associated with a favorable outcome, although late relapses are not infrequent.[71]

The role of 12p deletion, including the ETV6 gene, is not clear but it has been suggested as an important secondary event for the development of overt leukemia.[72] 12p deletions are also found in ALL patients with a normal karyotype and high diploidy, and in several solid neoplasias, suggesting a tumor suppressor gene is located in this region.[73]

RUNX1 rearrangements are detected in different hematopoietic malignancies and its protein binds to the promotor of many cell regulating genes important in hematopoiesis.[74] Fusion proteins resulting from RUNX1 translocation actively repress expression of a variety of target genes. Germline mutations of RUNX1 have been associated with rare cases of familial platelet disorder and with a predisposition to acute myelogeneous leukemia.[75]

BCR/ABL rearrangement

The t(9;22)(q34;q11), also called the Philadelphia (Ph) chromosome, occurs in about 3 % of childhood ALL with a higher incidence in adolescents, and in 25 % of adult ALL.[60, 76] The Ph chromosome is also seen in almost all children with CML, a rare leukemia disease in childhood representing 2-5 % of all cases.[77] This genetic translocation is associated with a very poor prognosis, and most children undergo HSC transplantation.[1, 78, 79]

The resulting gene rearrangement BCR/ABL, was the first fusion oncogene to be identified in leukemia and cancer. The resulting BCR/ABL fusion protein acts as an onco-protein with deregulated tyrosine kinase activity influencing several signaling pathways, contributing to the malignant transformation.[78] The breakpoints on chromosome 9 are usually scattered within the first intron of the ABL1 gene. The size of the BCR fragment differs, depending on splicing at different breakpoints clustered within two major areas of chromosome 22. In childhood Ph+ ALL 80-90% of the patients harbor the minor breakpoint cluster region (m-BCR) resulting in the formation of a 190 k Da fusion protein (p190). In CML major breakpoint cluster region (M-BCR), resulting in a protein of 210 kDa, is most common.[78] Both the major and the minor fusion proteins have enhanced tyrosine kinase activity and are located in the cytoplasm. Due to the heterogenous breakpoints, and that BCR/ABL is associated with adult leukemia it has not been investigated in neonatal DBS. A recent positive addition to the treatment of BCR/ABL positive leukemia is a tyrosine kinase inhibitor, imatinib.[23]

dic(9;20)

The dicentric chromosome abnormality dic(9;20)(p11-13;q11) was first reported as a non-random aberration in ALL 1995, and has been described as more common in

pediatric ALL (2%) than in adult ALL (<1%).[80, 81,82] The reason why dic(9;20) has not been detected earlier is that it is a subtle rearrangement, which by G-banding alone may be mistaken for monosomy 20 or deletion of 9p. Thus, FISH analysis is necessary for accurate identification of the abnormality (Figure 3).[83, 84]

Very little is known about the molecular consequences of dic(9;20), or of the type and frequency of additional genetic changes in pediatric ALL. The prognostic impact of dic(9;20) remains unclear and seems to vary between different treatment protocols.[81, 83] As the optimal treatment strategy for children with this aberration is unknown, the choice of treatment intensity has been made according to age, white blood cell count, the presence of extra-medullar leukemia, and response to induction treatment. Most patients have been allocated to non-standard risk treatment arms. Relapses are quite common but post-relapse treatment of many patients has been successful.[1]

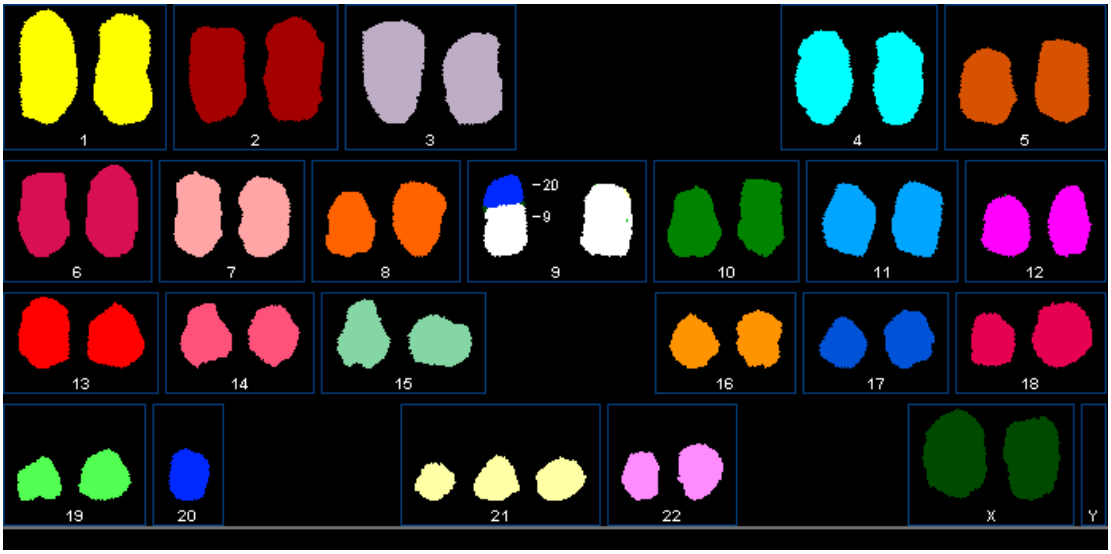

Figure 3. *SKY-imaging of the chromosomes from a leukemic cell positive for dic(9;20) and with trisomi 21.*

Cell cycle regulating proteins

Leukemia, like all forms of malignancies, is thought to be the result of genetic and possibly epigenetic changes that occur in stem or precursor cells. Two main categories of genes are involved in the process of carcinogenesis: oncogenes that are activated, and tumor suppressor genes that are inactivated by point mutation, deletion,

rearrangement, or duplication. Gene products of both types of genes are required for normal cell proliferation and differentiation, and aberrant expression leads to abnormal cell proliferation. Ras and p53 genes are the most well known examples of oncogenes and tumor suppressor genes, respectively.[85, 86] Since the elucidation of the mechanisms of mammalian cell division in the 1970s and 1980s, gene products that regulate the key cell cycle machinery have been investigated as cancer drug targets. Among these some target tyrosine kinases, such as the BCR/ABL protein kinase inhibitor imatinib which is used for the treatment of Ph-positive leukemia and CML.[87]

PTEN

PTEN (phosphatase and tensin homologue on chromosome 10) is a tumor suppressor gene involved in apoptosis, induction of cell cycle arrest, and regulation of cell adhesion, migration and differentiation. It is a negative regulator of the PI3K (phosphoinositide 3-kinase)-AKT pathway with a number of downstream targets that each influence cell cycle regulation or apoptosis and can contribute to the development of cancer.[88-91] PTEN protein product, a lipid phosphatase, antagonizes PI3K function and consequently inhibits downstream signaling through AKT promoting apoptosis and cell cycle arrest.

PTEN is commonly deleted or otherwise inactivated in diverse cancers such as melanoma, breast, gastric and prostatic carcinoma, and hematopoietic malignancies.[92-98] Germ line PTEN mutation is also associated with some human autosomal dominant cancer predisposition syndromes including Cowden disease, Bannayan-Zonana syndrome and Lhermitte-Duclos disease. [99-101] Loss of PTEN, even inactivation of one PTEN allele, allows AKT hyper-activation resulting in the inhibition of apoptosis and increased cellular proliferation. [89, 102, 103]

In ovarian cancer higher PTEN expression prolonged the time to progression and declining PTEN expression resulted in a shorter relapse-free interval. [104] In a study of Cowden syndrome, Trotman *et al*[105] has shown how ubiquination regulates PTEN nuclear import and tumor suppression. In Cowden syndrome PTEN is enzymatically functional but cannot be transported into the nucleus. Trotman found that transport of PTEN into the nucleus is essential for tumor suppression and nuclear import is mediated by monoubiquitination. Nuclear PTEN is stable and active (can inhibit AKT

and induce p53-independent apoptosis), while polyubiquination leads to its cytoplasmic retention and degradation. High levels of nuclear PTEN in Colon cancer were associated with a less advanced pathological stage.

Rapamycin, an immunosuppressant drug to prevent rejection in organ transplantations, stimulated apoptosis and increased doxorubicin-induced apoptosis in non-responder samples of childhood lymphoblastic leukemia cells.[106] Rapamycin can reactivate PTEN downstream activity by suppressing the mammalian target of rapamycin (mTOR) activity, one of the downstream targets of the PI3K-AKT pathway. Yilmaz et al[103] have shown, in a study with a conditionally PTEN depleted mouse model, that normal adult HSC and leukemic initiating cells (leukemia stem cells) employ different mechanisms for their self renewal. Depletion of PTEN led to a myeloproliferative disease that progressed rapidly to acute myeloic leukemia (AML) or ALL. Loss of PTEN also led to transient expansion but later depletion of the normal HSC pool. By administrating rapamycin to the PTEN depleted mice the leukemia-initiating cells disappeared and the normal HSC function was restored. Considering the finding of Yilmaz et al, of different mechanisms of PTEN in normal HSC and leukemic progenitor cells, this might be a novel approach to treatment of PTEN-depleted/inactivated leukemias.

SHP1

Another important cell cycle regulator is SHP1 (Src-homology 2-domain-containing protein tyrosine phosphatase), a cytoplasmic protein, working through the JAK/STAT, PI3K-AKT and other signal transduction pathways.[107] SHP1 is expressed primarily in hematopoietic cells and plays an important role in the differentiation, proliferation and activation of these cells by negatively regulating downstream many of the hematopoietic growth factor receptors. SHP1 terminates an activated signal by dephosphorylating molecules involved early in signal transduction.[108, 109]

Loss of SHP1 leads to upregulation of STAT resulting in decreased apoptosis, increased cell proliferation and promotes tumor genesis. SHP1 protein has been shown to be decreased or absent in leukemias and lymphomas and highly expressed in the mantle zone and interfollicular zone lymphocytes in reactive lymphoid hyperplasia.[110-112]

One of the mechanisms for SHP1 silencing is aberrant methylation of the promotor region, which has been shown in high frequency (75-100 %) in B-cell leukemia/lymphoma cell lines by Koyama *et al.*[110] Transfection of the intact SHP1 gene in those cell lines induced growth inhibition. Loss of heterozygosity (LOH) on chromosome 12, where the SHP1 gene is located, occurred in a high frequency in blast cells from patients with leukemia and loss of SHP1 expression was associated with a more aggressive disease.[111] Loss of SHP1 was observed in bone marrow biopsies of myelodysplastic syndrome (MDS) patients who progressed rapidly into acute leukemia [113] and in CML a decrease in SHP1 expression is associated with progression of the disease.[114] Thus silencing of SHP1 gene might be one of the critical events for the onset of malignant leukemia/lymphoma. It could also be an important diagnostic or prognostic marker and the target of gene therapy.

Src-kinases alter PI3K signaling pathway by inducing phosphorylation of specific substrates including PTEN. Phosphorylated PTEN is stabilized but inactive. SHP1 has the capability of restoring the function of PTEN by dephosphorylate PTEN, and thus decrease AKT activity. SHP1 alone does not influence AKT activity.[115] Phosphorylation of PTEN is associated with poor prognosis in AML.[116-118] In the genesis of leukemia depletion of SHP1 might cause lower degree of PTEN activity due to the lack of dephospholyration, and thus promote cancer progress. PTEN could then be highly expressed but phosphorylated and inactive.

VEGF, VEGFR-1 and VEGFR-2

Angiogenesis is a central regulator of cancer growth. The receptors on the endothelial cells are activated by proteins secreted by the tumor stimulating the growth of new blood vessels. Activated endothelial cells also produce cytokines inhibiting apoptosis.[119] Vascular endothelial growth factor, VEGF, is an angiogenetic factor involved in organ development, wound healing, tissue regeneration growth of endothelial cells and vessel permeability. VEGF exerts its effect by binding to its tyrosine kinase receptors. Receptor 1 and 2, VEGFR-1 and VEGFR-2, are present on the membrane of endothelial cells, hematopoietic cells and cancer cells.[120] VEGFR-1 promotes migration and proliferation of hematopoietic cells, while VEGFR-2 promotes proliferation of lymphatic endothelial cells as well as cell proliferation in

different cancers.[121, 122] There are autocrine positive loops, VEGF binding to VEGFR promotes an increase in VEGF levels and the signal is thus reinforced, which regulates hematopoietic stem cell survival.[123] Both of these receptors and VEGF have thus a key role in the pathology of bone marrow angiogenesis in leukemia.[124, 125]

VEGF has been reported as a putative biomarker and drug target, important in hematopoietic malignancies.[122] Increased micro vessel density in bone marrow samples from patients with acute or chronic lymphoblastic leukemia and AML is reported by several groups.[126-128] There is a positive correlation between VEGF expression and increased microvessel density in adult ALL and AML. High VEGF protein levels in serum and expression in bone marrow biopsies in adult ALL and AML cases were associated with worse prognoses.[126, 129]

Also in childhood ALL there is an increase of micro vessel density in the bone marrow and neovascularization in the bone marrow is associated with disease burden, prognosis and treatment outcome.[130, 131] Most Childhood ALL patients have detectable expression of VEGF in bone marrow or in peripheral blood, and increased levels of VEGF have been found to correlate with poor prognosis.[130, 132-134]

Treatment with drugs targeting VEGF or its receptors constitutes a new promising approach in the treatment arsenal of childhood leukemia.[87, 124, 125, 131] Among these new drugs are bevacizumab, an anti-VEGF monoclonal antibody, the first antiangiogenic therapy to be validated as a cancer drug, and sunitinib, a tyrosine kinase inhibitor specific for the VEGF receptor kinase.[119, 124]

The SLSG and NOPHO organizations

The Swedish pediatricians interested in the treatment of childhood acute leukemia formed in the late 1960s the Swedish Leukemia Study Group (SLSG) to make the diagnostic procedures and treatment of children with acute leukemia coordinated all over Sweden. They established a register including clinical parameters, immunophenotype, cytogenetic findings and outcome. The Nordic Society of Pediatric Hemathology and Oncology (NOPHO) started in the 1980s a register for all

five Nordic countries (Denmark, Norway, Finland, Iceland and Sweden) coordinating diagnostic procedures, treatment and follow up.

According to the new NOHPO ALL protocol from 2008 the child with a newly discovered leukemia is, based on bone marrow examination and peripheral white blood cell (WBC) count, placed in one of two induction groups. Depending on drug response and the cytogenetic examination the intensity of the following treatment is decided on day 29. At day 79 a last stratification takes place depending on MRD.[1] Children below the age of 1 year have a separate treatment protocol.

AIMS OF THIS STUDY

The general aim of this thesis has been to increase the understanding how genetic aberrations and alterations in cell cycle regulating proteins contribute to pathogenesis and prognosis in childhood ALL. More specifically the aims were:

– to investigate the incidence and to describe clinical and cytogenetic features of dic(9;20) in pediatric ALL.

– to investigate if the dic(9;20) and t(9;22) leukemic clone at diagnoses can be traced back to the time of birth.

– to investigate if the fusion proteins of ETV/RUNX1 and BCR/ABL at RNA level can be detected in the neonatal dried blood spots from children with leukemia.

– to investigate the expression levels of the cell cycle regulating proteins PTEN, SHP1, VEGF and VEGF receptor 1 and 2 in the leukemic clone at diagnosis and relapse.

MATERIALS AND METHODS

All information regarding materials and methods applied in this thesis has been described thoroughly in article I-VI. The studies were approved by the regional ethics committee and complied with the declaration of Helsinki on medical research involving human subjects.

Patients and samples

Article I and II

These studies were done on behalf of the NOPHO, the Swedish Cytogenetic Leukemia Study Group (SCLSG), and the NOPHO Leukemia Cytogenetic Study Group (NLCSG). The patients were recruited from the NOPHO database and diagnosed between 1996 and 2006. 1,827 infants, children, and adolescents were diagnosed with B-cell precursor (BCP) ALL in the Nordic countries (Denmark, Finland, Iceland, Norway, and Sweden) during this time period. All patients had been diagnosed and treated according to the closely related NOPHO-ALL 1992 and NOPHO-ALL 2000 protocols.[1] The dic(9;20) has not been a risk stratifying aberration in any of the two protocols.

Bone marrow was collected at diagnosis, and chromosome banding analyses were performed using standard methods in 15 cytogenetic laboratories in the Nordic countries. All abnormal karyotypes have been centrally reviewed annually (since 1996 in Sweden and since 2000 in all five Nordic countries). To retrieve as many dic(9;20)-positive ALL as possible, cases with either probable dic(9;20) or with abnormalities suggesting the presence of this aberration, such as monosomy 20 and deletion of 9p,[83] and with cell surplus to the initial cytogenetic investigations, were screened by FISH. In *article II* diagnostic bone marrow smears from 626 patients

were referred to our laboratory for investigation. Altogether, we obtained material and performed interphase FISH on more than a half (53%) of all Nordic pediatric patients diagnosed with ALL 2001-2006 (n=1174).

In *article I*, the Mitelman Database of Chromosome Aberrations in Cancer[82] was used to retrieve previously published pediatric (<18 years) dic(9;20)-positive BCP ALL. Data on age, gender, WBC, EFS, OS, and karyotypes, including findings from FISH and array CGH analyses, were extracted from the original articles.

In *article II* a total of 32 Nordic dic(9;20) positive BCP ALLs treated according to the NOPHO-ALL-2000 protocol were included in the statistical analyses. One Philadelphia-positive case with dic(9;20) was excluded from the 25 cases diagnosed by interphase FISH, using the novel three-step interphase FISH method detailed below. Eight additional cases were diagnosed by conventional cytogenetic analyses, including metaphase FISH, in the Nordic participating centers.

Article III and IV

Since 1974, almost all newborn children in Sweden undergo screening tests for severe inborn errors of metabolism. For this purpose, four spots of whole blood are blotted on filter paper (i.e. Guthrie cards) where a small amount is used for the analyses and the remaining spots are stored for future clinical or research use. This procedure is routinely used worldwide. Since 1981 the neonatal dried blood spots have been stored at a temperature of 4˚C and since 1996 in a controlled relative humidity not exceeding 30%.

To quantify the amount of RNA that could be eluted, filter papers with dried blood spots from the Swedish National PKU register at the Karolinska University Hospital Huddinge, stored for 1, 5, 10, 15 and 20 years, were randomly selected. For each time point five specimens were collected, and from each specimen five 3 mm punches of dried blood were used.

After patient and parental consent, neonatal blood spots from 18 children diagnosed with leukemia at Karolinska University Hospital (Stockholm, Sweden), Akademiska

Hospital (Uppsala, Sweden) or Umeå University Hospital (Umeå, Sweden) were retrieved. 8 of these children had BCP-ALL with a t(12;21), 4 had BCP-ALL with a dic(9;20) and 6 had a Philadelphia chromosome positive leukemia (3 ALL and 3 CML), cytogenetically characterized at diagnosis by routine analysis. A whole dried blood spot (14 mm in diameter, containing approximately 60 µL whole blood or $6x10^5$ lymphocytes) was available for all of the dic(9;20) specimen, and for half of the Ph+ and t(12;21) specimen. For the remaining specimen ½ a spot was used.

Article V and VI

Paraffin-embedded bone marrow tissues, fixed in formalin or Stieves solution, from 1982 to 1999 were obtained from the Karolinska University Hospital (Stockholm, Sweden) and Linköping University Hospital (Linköping, Sweden). The material used in *article V* included 23 diagnostic bone marrow samples obtained from children who remained in a relapse-free survival for more than 5 years after diagnosis (22 BCP-ALL and 1 T-ALL), and 8 samples taken at time of relapse (6 BCP-ALL and 2 T-ALL). In *article VI* the material included 31 diagnostic BCP- ALL bone marrow samples. Diagnosis and staging were made according to standard criteria of the NOPHO protocols. Clinical data were collected from patient records. In addition, 15 bone marrow samples were analyzed from children diagnosed with a nonmalignant disease who underwent bone marrow aspiration as part of the diagnostic procedure.

Methods

Chromosome analysis – G-banding

Routine cytogenetic analysis by G-banding was performed as a routine investigation on all patients included in this study as a part of the diagnostic procedure. Bone marrow cells are cultured and metaphases of acceptable quality are captured and analyzed in a computer aided karyotyping system (PSI) based on the unique chromosome banding. The method is limited to the rather low resolution of several megabases and the usually poor quality of the metaphase leukemic cells. It is the standard method to decide ploidy and to detect larger deletions and translocations.

Fluorescence in situ hybridization (FISH)

FISH has been widely applied in diagnostic and research settings since the first application in 1986.[135] This rapid and simple technique allows detection of specific numerical aberrations, gene rearrangements, deletions and amplifications on both smears and paraffin-embedded tissues. Fluorochrome-labelled DNA probes are hybridized with a complementary target DNA sequence in the interphase nuclei of cells or in metaphase spreads on a microscope slide. A fluorescent counterstain (DAPI) is added and the slide is viewed in a fluorescent microscope with an appropriate set of filters. Interphase FISH applied in the diagnosis of childhood leukemia allows for the detection of prognostic important aberrations not possible to analyze by G-banding or when the cell culture has failed. Another advantage is the possibility to study a large number of cells, increasing the detection rate of malignant clones.

In *article I and II* we used interphase FISH to detect the leukemic clones with a dic(9;20), which by G-banding alone can be mistaken for monosomy 20 with or without the deletion of the short arm of chromosome 9 (9p). We have developed a method to identify the dic(9;20) in interphase nuclei as well as methaphase chromosomes on diagnostic bone marrow smears by using a three step FISH approach. First a 9p21 probe was used in order to detect cases with deletion of 9p21. All cases with detectable homo or hemizygous 9p21 deletions were then investigated with probes specific to the p and q subtelomers of chromosome 20, in order to detect copy number imbalances between the p-arm and the q-arm on chromosome 20. Finally, in those sample with more 20p than 20q material, centromere 9 and centromere 20 probes were used in order to detect the dic(9;20) rearrangement. Interphase FISH was chosen since most of our material consisted of bone marrow smears. Additional metaphase FISH was performed in cases where cells in fixative were available.

RNA and DNA extraction from dried blood spots

By analyzing DNA from a sample it is possible to detect genetic aberrations with polymerase chain reaction (PCR) using probes specific for the genetic alteration you are interested in. However, the nucleotide base pair sequence of the aberration or the exact breakpoint of a translocation needs to be known. Often, in childhood leukemia,

the translocation results in a fusion gene, expressing mRNA for the fusion protein. In situations where the breakpoints are heterogeneous, and thus hard to detect by PCR on DNA-level, it is possible to trace the mRNA transcript for the fusion protein by analyzing RNA using reverse transcriptase creating cDNA and subsequent PCR. This method is routinely used in a clinical setting, checking for MRD by analyzing mRNA of the BCR/ABL fusion protein in bone marrow samples from children with a t(9;22) positive leukemia clone.

In *article III* RNA was isolated from the samples with the RNeasy micro kit (Qiagen) according to the manufacturer's instructions. Briefly, filters were incubated in lysis buffer at 37 °C for 30 min in a thermomixer (Eppendorf) at 1000 rpm. The lysates were applied to RNeasy columns, and after careful washing the RNA was eluted in 14 µL of RNase-free water. This eluate was reapplied twice to the column to maximize yield. 8 µL of the RNA was subjected to DNase I digestion and reverse transcribed using random hexamers. cDNA was detected by real-time quantitative PCR (ABI 7000 SDS, Applied Biosystems, Palo Alto, CA, USA) with oligonucleotides designed to amplify fragments of the mRNAs encoding human β-actin. For the purpose of these analyses threshold cycle (Ct) values were reported.

In *article IV* total RNA and genomic DNA were simultaneously purified from the samples with the Allprep DNA/RNA micro kit (Qiagen) according to the manufacturer's instructions. The filters were incubated in lysis buffer as described above, and the lysates were first passed through an AllPrep DNA spin column to selectively isolate DNA and then through an RNeasy MinElute spin column to selectively isolate RNA. In this way DNA and RNA are purified from the entire sample. DNA was eluted, after washing, twice in 30 + 30 µL buffer EB. RNA was eluted as described above.

Rea- time quantitative polymerase chain reaction (RQ-PCR)

In the PCR reaction it is possible to exponentially and selectively amplify specific regions of genomic DNA or RNA. RQ-PCR has the additional capability of accurately quantifying the nucleotide sequence of interest [136]. The TaqMan system is commonly utilized, including amplification with specific primers followed by the detection of emitted fluorescence from the hydrolyzed TaqMan dual-labeled probes.

In the analysis either DNA or RNA can serve as template, but the RNA strand needs to be reversed transcribed to complementary DNA (cDNA) prior to PCR amplification. Usually the term RT-PCR is used for real-time quantitative analysis when RNA is used as a template. The threshold cycle (Ct) value represents the number of PCR cycles necessary to detect a signal above the threshold and is inversely proportional to the amount of target present at the beginning of the reaction.

The amount of material needed for the analysis is small and the quality of the isolated nucleic acids is of great importance. RNA is extremely susceptible to degradation, thus careful pre-analytical handling of clinical samples is very important. In *article III* we determined the degradation of RNA purified from neonatal blood spots stored for up to 20 years. Amplifiable cDNA was detected by RT-PCR using primers specific for transcripts encoding β-actin, a common "housekeeping" gene.

In *article IV* we used neonatal blood spots from children diagnosed with leukemia. RNA was purified and, following reversed transcription by random hexamer primers into cDNA, investigated by RT-PCR using primers for ETV6/RUNX1 and BCR/ABL respectively. Purified DNA from the same samples is presently analyzed by RQ-PCR using primers for the specific IgH/TCR clone for the individual patient.

Tissue micro array (TMA) and immunohistochemistry

TMA allows the analysis of many samples on a single slide, eliminating glass to glass differences due to staining and other treatments (Figure 4). Very little material is needed, but the analysis could be biased due to the small sample and thus fewer cells. Before constructing the TMA in our study *(article V and VI)*, representative areas of hematoxylin and eosin-stained bone marrow sections were selected by an experienced hematopathologist. A manual arrayer was used and paraffin-embedded sections (4 μm) of all samples were placed on the same slide, deparaffinized and stained. Commercially produced monoclonal anti-human antibodies for PTEN, SHP1, VEGF-A, VEGFR-1 and VEGFR-2 were used for antigen detection. When the antibody binds to its corresponding protein it can be detected as a color change. In *paper VI* full tissue sections were prepared with the monoclonal antibodies for VEGF-A, VEGFR-1 and VEGFR-2 when no cells were identified for the specific sample in the TMA.

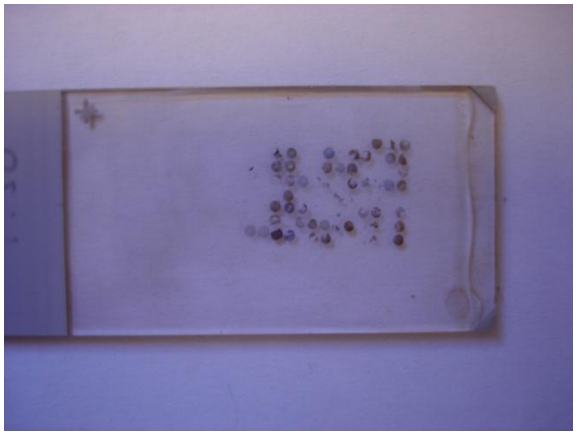

Figure 4. *Tissue micro array (TMA) with all samples on a single slide, allowing for simultaneous staining and treatment with the selected monoclonal antibody for immunohistochemistry.*

The staining intensity of the antibodies on leukemic blasts or normal mononuclear cells, reflecting the amount of protein present in the cell, was examined in a light microscope. At least 200 mononuclear cells were counted and the results were interpreted without knowledge of the clinical information. This method relies on the specificity of the antibodies and there is always the risk of unspecific binding. The staining intensity is a crude measurement of the expression levels of the protein in the cell and it is possible to identify the location of the protein in the cytoplasm or the nucleus. However, in order to determine the functionality of the protein, if it is active or not, other methods are necessary.

Statistical analysis

Article I and II

The probability of EFS and OS was calculated using the Kaplan-Meier method and the different cytogenetic and clinical subgroups were compared using the log rank test. The significance limit for p-values was set to 0.05 in all tests. The Chi-square test with exact calculation of p-value was used to investigate possible correlations between cytogenetic groups and clinical characteristics. Multivariate analysis using Cox regression model was performed to identify cytogenetic and clinical factors that independently had an impact on EFS and OS. In the analysis of EFS, events comprised induction failure, relapse, death in remission, and second malignancy. In the OS analysis, death was the endpoint.

Article III

A one way ANOVA was performed to test if there were any difference between each eluate based on ct values from the RT-PCR and total yield of RNA calculated based on spectrophotometric reading. A P-value of less than 0.05 was considered statistically significant.

Article V

Logistic Regression was used to discriminate between expression of PTEN and SHP1 in the diagnostic leukemia group and the non-malignant group. The two factors were tested univariate. Agreement between observed and predicted was estimated as percentage agreement. The test was two sided. P-values of less than 0.05 was considered statistically significant.

Article VI

Mann-Whitney U test or Spearman rho test was used to examine the statistical significance of associations between VEGF, VEGFR-1, VEGFR-2 and the clinical variables. The Mann-Whitney U test was used to verify statistical correlations of the two clinical groups and the protein expression. To analyze significant associations between VEGF and its receptors within the two clinical groups Spearman rho test was employed. P-values less than 0.05 were considered statistically significant.

RESULTS

Article 1

A total of 71 pediatric (<18 years) BCP ALLs with dic(9;20) were available for analysis, 24 newly identified patients from the Nordic countries and 47 from the literature. Out of 1,827 pediatric BCP ALL diagnosed in the Nordic countries 1996–2006, G-banding together with FISH analyses identified the dic(9;20) in 24 (1.3%). The most common modal chromosome numbers were 45 and 46. No case had less than 44 and no case had more than 50 chromosomes. Aberrations in addition to dic(9;20) were found in 63%, and among the cases with additional changes, the vast majority (84%) had unbalanced aberrations only. The most common cytogenetically identified imbalances were gains of chromosomes 21 (20/71; 28%) and X (7/71; 10%).

In the total cohort of 71 patients, the median age was 3 years and the female to male ratio was 2.0. The age specific incidence pattern was similar to that earlier found in all pediatric ALLs, but the peak was observed at a somewhat earlier age (1–3 years). Very few cases have been reported above the age of 5. Few cases were reported to have extra-medullar leukemia, with CNS involvement in 4/38 (11%) and a mediastinal mass in 2/38 (5%) cases being the most common. The median WBC count was 24 x 10^9/L. Data on risk stratification had been given for 15 previously reported cases and for all Nordic cases, where 31/39 (79%) cases were nonstandard risk. The predicted EFS and OS at 5 years for the 24 Nordic cases was 0.62 and 0.82, respectively.

10 Nordic cases were investigated with FISH for CDKN2A, and homozygous loss of this gene was found in one case, the remaining nine tested cases had, as expected, hemizygous deletions. Eight of the 49 previously reported cases had been analyzed by FISH for CDKN2A alterations, and five of these had homozygous deletions. Thus, 6 (33%) of 18 dic(9;20)- positive ALL tested displayed homozygous loss of CDKN2A.

Article II

Among 626 patients referred to our laboratory, diagnosed between 2001 and 2006, 542 (86%) were diagnosed with BCP-ALL and 81 (13%) had T-cell ALL (in three patients no information about immunophenotype was available). In the T-ALL samples deletion of 9p21 was found by interphase FISH in 33 cases (41%), and further investigation found no case with dic(9;20).

A total of 542 patients with BCP-ALL patients were analyzed with interphase FISH, 93 had a deletion of 9p21, and in total dic(9;20) occurred in 4.6% (25/542). In the 32 cases diagnosed between 2001 and 2006, and enrolled in the NOPHO-ALL 2000 protocol, the predicted EFS at 5 years was 0.68. The predicted EFS for dic(9;20) was significantly worse compared to the other two large groups of leukemia within the childhood peak, the t(12;21) and the group with HeH. The predicted OS at 5 years was 0.83, which was significantly worse compared to cases with with t(12;21) but no significant differences were found when compared to the HeH subgroup. The OS in different cytogenetic subgroups in the cohort of 542 BCP-ALLs included in this study are shown in Figure 2 (page 20).

Article III

Transcripts encoding human β-actin were detected in all 25 samples analyzed at a mean threshold cycle (Ct) of 25 (SD 1.9). A one way ANOVA indicated no significant effect of storage time on Ct values ($p>0.05$), suggesting that significant RNA decay did not occur over the period of 20 years studied.

Article IV

Transcripts for ETV6/RUNX1 and BCR/ABL could not be detected by RT-PCR, using clone specific primers, in neonatal blood spots from children with the leukemic clone at diagnosis. Analyzing the DNA for clone specific IgH/TCR rearrangements for dic(9;20) and t(12;21) positive samples is currently ongoing.

Article V

PTEN was significantly over expressed in diagnostic ALL samples, compared to the nonmalignant group. Expression of PTEN was seen in 21/22 (96%) ALL samples and

mean proportion of positively stained cells was 78%. In the nonmalignant group PTEN was expressed in all cases, but the mean proportion of positively stained cells was 39.5%.

SHP1 showed a significantly lower expression in the diagnostic ALL samples than in the nonmalignant group. Expression of SHP1 was seen in 5/24 cases (21%) at diagnosis of ALL and mean proportion of positively stained cells was low, only 6%. In the nonmalignant samples SHP1 was expressed in 6/10 cases (60%) with a higher mean proportion of positively stained cells of 24.5%.

In the samples taken at relapse of the disease expression of PTEN was observed in 4/5 (80%) and all samples expressed SHP1, mean proportion of positively stained cells was 40% and 31 %, respectively. The group was too small to allow meaningful statistical analysis.

Article VI

The proteins VEGF, VEGFR-1 and VEGFR-2, were expressed in the majority of leukemic bone marrow samples: VEGF in 92.9%, VEGFR-1 in 80.6%, and VEGFR-2 in 56.0%. In the non malignant group the majority of samples displayed a positive staining for VEGF (76.9%), while only 35.7 % of the samples expressed VEGFR-1 and 40 % VEGFR-2. ALL patients had significantly increased expression of VEGFR-1 compared to the nonmalignant group.

Parameters including risk group, age, sex, WBC count, and status of bone marrow transplantation in patients with high expression of these proteins were compared with patients with low or negative expression. No association was observed between the expression intensity of the angiogenic proteins in the malignant group and clinical parameters. In addition, we investigated if VEGF expression was correlated with expression of its receptors. No correlations were observed between the expression of VEGF, VEGFR-1, and VEGFR-2.

DISCUSSION

The dic(9;20) aberration (Article I, II)

We have found that dic(9;20) occurs in 4.6% of childhood BCP-ALL, a figure more than twice as high as previously reported[137] and making it the third most common genetic subgroup of BCP-ALL after HeH and t(12;21)(p13;q22). Most of the earlier analysis have been based on karyotyping where monosomy 20 has been used as a pointer to dic(9;20). Approximately 50% of all dic(9;20) cases will then escape detection, since many cases have two normal chromosomes 20 in addition to the dic(9;20). Our previous FISH analyses and array-CGH studies have revealed that the dic(9;20) contains centromeres of both chromosomes 9 and 20, and results in loss of 9p and 20q material.[138] We have developed a robust method where it is possible to detect the aberration on bone marrow smears, in a routine clinical setting, using a three step FISH approach. Using this method there is a very small risk that cases with dic(9;20) would escape detection and we therefore believe that our incidence figure is true.

Even though most children with ALL now can be cured, the prognosis is still more dismal for about 20 % of the children who have relapsing disease. Our study, and several other reports, strongly indicate that a substantial proportion of the dic(9;20) positive cases relapse and that there is an increased risk for extramedullar disease and CNS relapse. It is therefore very important to identify this subgroup of leukemia in order to assign the patients to correct risk groups and proper treatment regimens. As a result of our studies, patients with dic(9;20) are now stratified to the intermediate risk group in the NOPHO-ALL 2008 protocol, in use since July 1 2008 within the Nordic countries.[1]

In a recent report by Pichler *et al*,[139] 19 dic(9;20) positive BCP-ALLs treated according to the ALL Berlin-Frankfurt-Munster (BFM) protocols including an intensive induction and post-induction consolidation therapy showed a rather

favorable outcome with a lower relapse rate (16 % vs 29%) and a higher 5-year EFS rate estimate (75 % vs 62 %) compared to the outcome for the NOPHO patients. This strongly emphasizes that the prognostic relevance of a dic(9;20) has to be evaluated in the context of the chemotherapeutic protocols applied. Since in vitro studies have shown high cellular sensitivity of dic(9;20)-positive leukemic blasts to l-asparaginase and cytarabine,[140] Pichler *et al* speculate that both drugs, when given early during BFM-like induction and consolidation therapy, may have contributed to the good outcome in patients treated by the BFM protocol. This study indicates that relapses in BCP-ALL with a dic(9;20) might be prevented by a BFM-like time- and dose-intensified 4-drug induction and subsequent consolidation therapy.

Even though dic(9;20) can occur together with other prognostically important aberrations, such as t(9;22), t(12;21) and high hyperdiploidy, the fact that dic(9;20) is the sole change in about 40% of the cases, strongly suggests that it may be considered a primary abnormality in childhood BCP ALL. Indirect support for this conclusion is the finding that the vast majority of the additional changes are genomically unbalanced, which is typical for secondary neoplasia-associated abnormalities.[141] In contrast to most other primary leukemia-associated aberrations,[142] the dic(9;20) does not seem to result in a fusion gene.[138, 143] Instead, loss of genetic material may be the functionally important outcome. It is in this context noteworthy that CDKN2A gene was homozygously deleted in one third of all cases analyzed by FISH.

It is of utmost importance to study larger series of patients and to develop routines for the detection and optimal treatment of this highly under-diagnosed rearrangement. Further understanding of the implications of the genetic changes in dic(9;20), will hopefully reveal candidate genes involved in the leukemogenic process. Detailed characterization of the breakpoints in our group has showed clustering of breakpoints on both chromosome 9 and 20, but no common breakpoint region was found.[138] Thus, it remains to be elucidated whether dic(9;20) leads to a chimeric gene or whether the functionally important outcome is loss of 9p and 20q material. In addition, the reason why dic(9;20) is twice as common in female than male children needs to be investigated.

Tracing back to birth (Article III and IV)

We report that transcripts encoding β-actin are readily detectable in RNA isolated from neonatal DBS stored for up to 20 years. Using RT-PCR and spectrophotometric analysis, we have found that no significant loss of transcripts appeared to occur over time. These results are consistent with previous studies, where the amount of preserved RNA in up to 9 years old samples has been quantified [144].

Many diseases with an onset during childhood or in adult life have their origin in events taking place during fetal life or at birth. Since the neonatal blood spots are collected prospectively with no selection bias and cover almost the entire population, they represent an invaluable resource for epidemiological research. Also, in combination with patient registers they constitute a precious tool in the search of the molecular basis of a disease. The range of analytical possibilities is, however, limited by the small amount of blood available. Several research groups have recovered DNA from these filter papers after long term storage, with subsequent molecular analyses including whole genome amplification and detection of specific mutations or genetic rearrangements.[36, 145, 146] Being able to also use retrieved RNA from these neonatal DBS, opens new possibilities to investigate the origin of several diseases where RNA of a protein of interest is known, but the specific DNA sequence is unknown.

Stored neonatal dried blood spots from children who have developed leukemia, have been analyzed for chromosomal aberrations found at diagnosis, by sequencing the unique patient gene rearrangement in the leukemic clone and determine if the sequence was present in the DNA isolate.[33, 35-37] In leukemia cases lacking specific chromosomal translocations, analysis of clone-specific immunoglobulin heavy chain (IgH) and T-cell receptor (TCR) gene rearrangements have provided indirect support for a prenatal origin.[41, 43]Mori *et al*[45] were able to detect ETV6/RUNX1 transcript in 6 out of 567 (1%) cord blood samples, a frequency that exceeds by a factor of 10-20 the cumulative risk of leukemia, suggesting that the preleukemic clone is functional at birth and expresses the protein. However, in another series that investigated the presence of ETV6/RUNX1 transcripts in cord blood samples from premature children no positive sample could be detected amongst 256 samples.[147]

We were unable to detect transcripts for ETV6/RUNX1 and BCR/ABL fusion genes by quantitative RT-PCR in neonatal DBS from children with the leukemic clone at diagnosis. One possible explanation might be that the amount and integrity of the RNA were not enough to enable detection of low levels of transcript. Based on ETV6-RUNX1 expression data derived from Gabert *et al*,[148, 149] the current assay should be able to detect 1 ETV6-RUNX1 positive cell in a background of 10^5-10^6 cells. Assuming equivalent expression in the preleukemic ETV6-RUNX1 positive cells and considering the amount/integrity of the RNA isolated from the dried blood spots, ETV6-RUNX1 is either not expressed at all in the preleukemic cells or only expressed at levels below those corresponding to 10^{-4} cells for the best preserved samples and below $5x10^{-2}$ for the most degraded samples. In a pair of monochorionic twins, where one twin developed ETV6-RUNX1 positive preB ALL, ETV6-RUNX1 transcript expression was detected in rare CD34+/CD38-/CD19+ cells but not in progenitor B cells from the healthy twin.[150] Our negative results could also reflect the possibility that the amount of extracted RNA was too low to allow the detection of the rare CD34+/CD38-/CD19+ cells expressing the fusion transcript.

The Philadelphia chromosome positive leukemia has a higher incidence in adolescents and adults, and the presence of the clone has previously not been assessed in neonatal dried blood spots. We investigated 6 filters with dried blood from patients who later developed a Philadelphia positive leukemia and all were negative for the fusion transcript. There is evidence the t(9;22) is both necessary and sufficient for CML induction[151] and it is thus possible this leukemia fusion has its origin in events taking place after birth also in childhood leukemia. BCR-ABL mRNA is detectable in the blood of a high proportion of normal adults, but in very low copy number suggesting that these rearrangements are almost certainly not arising in the stem cell compartment with consequent clonal expansion.[45] In conclusion, further studies with larger series of patients are necessary to investigate the pathogenesis of Philadelphia positive leukemia.

The patients in our material of dic(9;20) positive leukemia had a clear incidence peak at 1–3 years, thus contributing, together with t(12;21) and high hyperdiploidy,[1] to the characteristic childhood peak of BCP ALL. Considering the findings that both t(12;21) [33, 35, 36, 45] and high hyperdiploidy [39-41] often arise *in utero*, it is tempting to

speculate that also dic(9;20) has a prenatal origin. We are presently investigating the indirect presence of dic(9;20) in neonatal blood spots by RQ-PCR using primers for clone specific IgH/TCR rearrangements.

Cell cycle regulating proteins (Article V and VI)

We found a higher expression of PTEN and VEGFR-1, and a lower expression SHP1 in pediatric ALL patients at diagnosis compared with pediatric patients with a non-malignant disease. It should be noted, however, that our sample group is small and it is therefore difficult to draw any firm conclusions from the findings.

The network on how cell cycle regulating proteins work, with activating receptors for enhancing or inhibiting a downstream signal or transportation into the nucleus, is very complicated and only partially understood. A further complication is that the protein might be expressed in the cell but inactivated, or not able to enter the nucleus to exert its effect. We know some of these proteins are involved in the malignant transformation of a cell-clone, by altered expression or changed activity due to genetic and cell signaling changes. PTEN has been shown depleted or inactivated in various cancers [92-98]. Yang *et al* [152] have in blast cells from 8 pediatric ALL patients seen a low expression of PTEN, and Roman-Gomez *et al* [96] have shown that the PTEN promoter was methylated, and thus silenced, in 20% of pediatric ALL. SHP1 has in adult leukaemias and lymphomas shown a variable expression correlated with gene promoter methylation, and treatment of B-cell lines with a demethylating agent resulted in re-expression of SHP1 protein.[110-112] VEGF has been reported as a putative biomarker important in hematopoietic malignancies, where increased micro vessel density has been correlated to VEGF expression levels in ALL.[122, 126, 129]

Contradictory to previous reports we found PTEN highly expressed at diagnosis. These diagnostic bone marrow samples were from children with a 92 % 5-year disease-free survival. In the relapse samples there was a trend of lower expression of PTEN, but the group was too small for meaningful statistical analysis. A high expression of PTEN could indicate a less malignant disease, and PTEN could thus be a possible candidate for a prognostic marker. Yilmaz *et al*'s[103] PTEN-depleted mouse model indicate different roles for PTEN in malignant and normal HSC. The leukemia-initiating cells disappeared and the normal HSC function was restored when

PTEN downstream pathways was reactivated by rapamycin. This might be a novel approach to treatment of PTEN-depleted/inactivated leukemia. In our study the antibodies were selective for total PTEN and it was not possible to differ between active or inactivated PTEN. If PTEN is used as a prognostic marker or PTEN activity is reactivated as a part of the treatment, there is a need to be able to identify PTEN activity. Phosphorylated PTEN is stabilized but inactive and SHP1 has the capability of dephosphorylating PTEN, thus restoring its function.[115] Low levels of SHP1 might reflect low activity of PTEN, if there is a process going on phosphorylating PTEN.

Several studies of adult hematological diseases have seen an association between decreased expression of SHP1 and a more advanced disease.[113,114] The lower expression of SHP1 in the diagnostic group compared to non-malignant controls is supporting previous observations, suggesting loss of SHP1 to be a part of the hematological malignant transformation.[110-112] There was a trend to a higher expression in the relapse group. However, a relapsed leukemia has a history of treatment with several cell toxic drugs that might influence apoptosis and thereby up regulate SHP1.

Future studies are needed to further investigate the role and expression of PTEN, SHP1, VEGF and its receptors in pediatric leukemia. We need to determine if the expressed proteins are active or not, and if the expression levels are correlated with prognosis. A further understanding of cell cycle regulatory mechanisms and pathways in which the proteins operates could in lead to a novel approach in treatment of childhood leukemia with reactivation of the protein or downstream pathways.

SVENSK SAMMANFATTNING

Behandling av barn med leukemi tillhör en av de största framgångarna inom modern medicinhistoria. Från att ha varit en så gott som 100 % dödlig sjukdom för bara ett halvt sekel sedan, överlever nu nära 90 % av barnen med akut lymfatisk leukemi (ALL). ALL är den vanligaste cancerformen hos barn och utgör 80 % av barnleukemierna. Sjukdomen karakteriseras av en ackumulering av maligna, omogna och icke funktionella vita blodkroppar i benmärg och perifert blod. Dessa maligna celler tränger ut de normala blodkropparna och patienten har ökad risk att få infektioner, blodbrist och blödningar. Leukemi beskrevs först på mitten av 1800-talet, och kallades redan då *weisses Blut* (vitt blod), vilket leukemi ordagrant betyder. Det var först på 40-talet som mediciner började utvecklas, så kallade cellgifter eller cytostatika, som fick leukemi-patienter att tillfälligt bli bättre, men de återföll dock nästan alltid i sin sjukdom. På 60- och 70-talen blev behandlingen effektivare genom kombination av olika cytostatika, längre behandlingstider och barnen erhöll högre doser. Genom att identifiera olika markörer vid diagnos som påverkar prognosen – såsom ålder, antal vita blodkroppar samt morfologiskt och genetiskt utseende hos leukemicellen – började man också dela in patienterna i riskgrupper, så att de med en högrisk-leukemi får en mer intensiv behandling än de som saknar riskmarkörer. Barn-leukemicentra började också alltmer samarbeta, så att man mer systematiskt och vetenskapligt kunde utarbeta behandlingsprotokollen och sedan utvärdera dessa. Behandling som anpassas efter kända riskgrupps-markörer, kombinerat med vår ökade kunskap om att bemästra tillstötande infektioner, har starkt bidragit till behandlingsframgångarna vid barnleukemi.

Leukemicellen har förvärvat ett antal genetiska förändringar, vilka ändrar dess egenskaper vad gäller tillväxt, utmognad och överlevnad. Ofta resulterar sådana genetiska förändringar i att uttrycket av proteiner som reglerar cellens delning, s k cellcykel-reglerande proteiner, påverkas. Idag är vissa genetiska förändringar i leukemicellen och patientens initiala svar på behandlingen våra viktigaste

prognosmarkörer för att placera barnet med leukemi i rätt behandlingsgrupp. Återfall i sjukdomen är dock fortfarande den vanligaste orsaken till att barnet inte överlever. Det finns därför behov av att hitta nya markörer för prognos och att bättre anpassa behandlingen för de barn som har hög risk att få återfall i sin sjukdom.

Syftet med den här avhandlingen var att öka kunskapen om hur genetiska förändringar och cellcykel-reglerande proteiner vid ALL hos barn, bidrar till sjukdomens uppkomst och prognos.

I de första två studierna undersökte vi förekomsten av en genetisk förändring där material från korta armen på kromosom 20 har flyttat till kromosom 9, och material från korta armen på kromosom 9 och långa armen på kromosom 20 har gått förlorat. Denna nya kromosom som förekommer vid framför allt barnleukemi kallas dic(9;20), och den har tidigare med rutinmetoder varit svår att upptäcka och förekomsten har därför varit underskattad. Vi har undersökt sparat material från benmärgsprover som togs vid diagnos från 542 patienter från Norden som insjuknat 2001-2006, vilket är ungefär hälften av alla barn som fick ALL under denna period. Vi har utvecklat en metod att säkert identifiera om leukemicellerna bär på dic(9;20) genom att i 3 steg använda en metod som kallas FISH, fluorescent *in situ* hybridisering. Vid FISH använder man sig av prober som fäster på specifika ställen i genomet. När proberna är fästa vid DNA-strängen lyser de med en färg som kan ses i fluorescerande ljus i ett särskilt mikroskop. Vi har först använt prober för att identifiera patienter som saknar material från korta armen på kromosom 9, och därefter identifierat de med mer material från korta armen än långa armen på kromosom 20. Slutligen har vi använt prober för centromererna på kromosom 9 och 20, dic(9;20) innehåller båda centromererna. För att få fram kliniska parametrar har vi utnyttjat NOPHO (Nordic society of pediatric hematology and oncology)-databasen. Vi har också sammanställt tidigare publicerade fall där dic(9;20) finns beskrivet vid barnleukemi.

Vi fann att 4,6 % (25/542) av de barn som insjuknar i ALL har dic(9;20), vilket är dubbelt så vanligt än man tidigare trodde. Detta gör dic(9;20) till den tredje vanligaste genetiska förändringen vid barn-ALL, efter höghyperdiploid (när cancercellerna innehåller ett stort antal extra kopior av vissa kromosomer) ALL och ALL med t(12;21) (material från kromosom 21 har satt sig på kromosom 12). Återfall i

sjukdomen var relativt vanligt för patienterna med dic(9;20) och 5-års överlevnaden var 83 %, vilket är signifikant sämre än för de med t(12;21). Flickor med dic(9;20) var dubbelt så många som pojkarna och medianålder var 3 år, vilket är något yngre än för alla barn-ALL. De andra vanliga genetiska förändringarna i denna åldersgrupp uppstår ofta redan i fosterlivet, varför det är möjligt att även dic(9;20) gör det. Vilken påverkan dic(9;20) har på cellens uttryck av cellcykelreglerande proteiner, d v s hur den påverkar eventuella oncogener eller tumörsupressorgener, är ännu inte känt. Sammanfattningsvis har vi visat att dic(9;20) är dubbelt så vanligt förekommande mot tidigare uppfattning och att det är en genetisk förändring med ökad risk för återfall, vilket innebär att patienten inte bör få standardbehandling. Från och med NOPHOs nya behandlingsprotokoll ALL-2008, finns dic(9;20) med som en riskmarkör för att få en mer intensiv behandling.

I studie III och IV har vi undersökt filterpapper med torkat blod från neonatalperioden, s k PKU-prover. Genom att utvinna DNA ur det torkade blodprovet från födelsen kan man undersöka om specifika genetiska avvikelser uppstår under fosterlivet. Andra forskargrupper har i nyföddhetsprovet identifierat sådana specifika genetiska förändringar från leukemiklonen vid diagnos. Man tror därför att ett förstadie till leukemin, där cellerna har vissa genetiska förändringar, ofta uppstår under fosterlivet. Ytterligare genetiska avvikelser ackumuleras sedan under barndomen, innan leukemin bryter ut. Vi har undersökt om även RNA finns bevarat i det torkade blodprovet och om det är bevarat över tiden. Vi fann att RNA kan utvinnas ur upp till 20 år gamla torkade blodprover från födelsen, och att det tycks välbevarat även i de äldsta proverna. Fördelen med att använda RNA, istället för DNA, i analyser är att man inte behöver känna till den exakta DNA-sekvensen och att mycket lite material krävs. Dessa sparade PKU-prover kan därför användas för att undersöka uppkomstmekanismer för sjukdomar som har sitt ursprung före födelsen, men som bryter ut först senare under barndomen eller vuxenlivet.

Vi har också undersökt torkade blodprover från födelsen från 14 barn som senare utvecklade leukemi och lika många friska kontroller. 8 barn var diagnosticerade med t(12;21) positiv ALL som ger upphov till fusionsproteinet ETV6/RUNX1, och 6 barn hade leukemi med den s k Philadelphia-kromosomen, (t(9;22), som ger upphov till fusionsproteinet BCR/ABL. Vi ville undersöka om RNA från fusionsproteinet kunde

påvisas i PKU-proven. Barn med Phildelphia-positiv leukemi är ofta något äldre, och den är vanligare hos ungdomar och vuxna. Det är tidigare inte undersökt huruvida Philadelphia-klonen kan återfinnas i PKU-provet, men man tror att denna genetiska förändring uppstår närmare diagnos eftersom djurförsök visat att förändringen ensam kan orsaka leukemi. När det gäller t(12:21) har flera grupper tidigare visat på DNA-nivå att förändringen uppstår under fosterlivet. Djurförsök har också visat att t(12,21) inte ensam orsakar leukemi, utan att fler genetiska förändringar krävs innan sjukdomen blir manifest. I vår studie kunde RNA framgångsrikt utvinnas ur samtliga PKU-prover, men RNA-transkript för ETV6/RUNX1 eller BCR/ABL kunde inte påvisas i något prov med hjälp av real-tids PCR (*polymeras chain reaction*). Analyser pågår för att se om dic(9;20) kan finnas redan vid födelsen. Vi tittar då indirekt efter förändringen på DNA-nivå genom att identifiera de specifika immunoglobuliner leukemiklonen uttrycker, eftersom vi inte känner till något fusionsprotein och det är arbetsamt att kartlägga den exakta DNA-sekvensen för varje patient.

I arbete V och VI har vi undersökt förekomsten av vissa cellcykel-reglerande proteiner, som man vet har en roll när en cell utvecklas till en cancercell, i benmärgsmaterial från barn med ALL. Uttryck av sådana proteiner skulle i en framtid kunna användas som prognosmarkörer för sjukdomens allvarlighetsgrad eller när man försöker identifiera återfall i sjukdomen. Vi undersökte proteinerna PTEN, SHP1, VEGF samt receptor 1 och 2 för VEGF (VEGFR-1 respektive VEGFR-2) i benmärgsmaterial från barn med ALL, och hade som kontroller benmärgmaterial från barn med icke-maligna sjukdomar eller friska benmärgsdonatorer. Vi använde oss av immunohistokemi, där specifika antikroppar fäster vid ett visst protein i cellen och då färgar det området i cellen. Med hjälp av ”tissue micro array” (TMA), kan ett större antal benmärgspreparat analyseras samtidigt på ett objektsglas. I mikroskop räknar man hur stor andel av cellerna som är färgade med antikroppen.

Våra resultat visade att PTEN överuttrycks medan SHP1 har ett lågt uttryck i diagnostiska ALL prover jämfört med icke-maligna kontroller. ALL-material hade också ett högt uttryck av VEGFR-1 jämfört med kontrollerna, som inte alls uttryckte proteinet, vilket kan tyda på ett samband mellan VEGFR-1-uttryck och ALL. Vårt material var relativt litet, varför det är svårt att dra några slutsatser. För att kunna

utröna dessa proteiners roll vid barn-ALL är det nödvändigt med fler och större studier.

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REFERENCES

1. Schmiegelow K, Forestier E, Hellebostad M, Heyman M, Kristinsson J, Soderhall S, et al. Long-term results of NOPHO ALL-92 and ALL-2000 studies of childhood acute lymphoblastic leukemia. *Leukemia*. 2010;24(2):345-354.
2. Piller GJ. Leukaemia - a brief historical review from ancient times to 1950. *British Journal of Haematology*. 2001;112(2):282-292.
3. Gilgenkrantz S, Rivera EM. The history of cytogenetics - Portraits of some pioneers - Thomas Hunt Morgan. *Annales De Genetique*. 2003;46(4):433-442.
4. Smeets D. Historical prospective of human cytogenetics: from microscope to microarray. *Clinical Biochemistry*. 2004;37(6):439-446.
5. Nowell PC, Hungerford DA. Minute chromosome in human chronic granulocytic leukemia. *Science*. 1960;132(3438):1497-1497.
6. Casperss.T, Zech L, Johansso.C, Modest EJ. Identification of human chromosomes by DNA-binding fluorescent agents. *Chromosoma*. 1970;30(2):215-&.
7. Rowley JD. New consistent chromosomal abnormality in chronic myelogenous leukemia identified by quinacrine fluorescence and giemsa staining. *Nature*. 1973;243(5405):290-293.
8. Rudkin GT, Stollar BD. High-resolution detection of DNA-RNA hybrids insitu by indirect immunofluorescence. *Nature*. 1977;265(5593):472-473.
9. Kallioniemi A, Kallioniemi OP, Sudar D, Rutovitz D, Gray JW, Waldman F, et al. Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. *Science*. 1992;258(5083):818-821.
10. Schrock E, duManoir S, Veldman T, Schoell B, Wienberg J, FergusonSmith MA, et al. Multicolor spectral karyotyping of human chromosomes. *Science*. 1996;273(5274):494-497.
11. Speicher MR, Ballard SG, Ward DC. Karyotyping human chromosomes by combinatorial multi-fluor FISH. *Nature Genetics*. 1996;12(4):368-375.
12. Nurse P, Masui Y, Hartwell L. Understanding the cell cycle. *Nature Medicine*. 1998;4(10):1103-1106.
13. Nurse P. Cell-cycle control in yeasts. *Biochemical Society Transactions*. 1977;5(4):1191-1193.
14. Hartwell LH, Culotti J, Pringle JR, Reid BJ. Genetic-control of cell-division cycle in yeast. *Science*. 1974;183(4120):46-51.
15. Masui Y, Markert CL. Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. *Journal of Experimental Zoology*. 1971;177(2):129-&.
16. Evans T, Rosenthal ET, Youngblom J, Distel D, Hunt T. Cyclin - a protein specified by maternal messenger-RNA in sea-urchin eggs that is destroyed at each cleavage division. *Cell*. 1983;33(2):389-396.
17. Martin GS. The road to Src. *Oncogene*. 2004;23(48):7910-7917.
18. Rowley JD. Human oncogene locations and chromosome-aberrations. *Nature*. 1983;301(5898):290-291.
19. Alitalo K, Schwab M, Lin CC, Varmus HE, Bishop JM. Homogeneously staining chromosomal regions contain amplified copies of an abundantly

expressed cellular oncogene (C-MYC) in malignant neuroendocrine cells from a human-colon carcinoma. *Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences*. 1983;80(6):1707-1711.

20. Pinkel D. Treatment of acute lymphocytic-leukemia. *Cancer*. 1979;43(3):1128-1137.
21. Thomas ED. Stem-cell transplantation - past, present and future. *Stem Cells*. 1994;12(6):539-544.
22. Weiden PL, Flournoy N, Thomas ED, Prentice R, Fefer A, Buckner CD, et al. Anti-leukemic effect of graft versus host disease in human recipients of allogeneic marrow grafts. *New England Journal of Medicine*. 1979;300(19):1068-1073.
23. Pui CH, Jeha S. New therapeutic strategies for the treatment of acute lymphoblastic leukaemia. *Nature Reviews Drug Discovery*. 2007;6(2):149-165.
24. Pui CH, Evans WE. Treatment of acute lymphoblastic leukemia. *N Engl J Med*. 2006;354(2):166-178.
25. Pui CH, Robison LL, Look AT. Acute lymphoblastic leukaemia. *Lancet*. 2008;371(9617):1030-1043.
26. Hjalgrim LL, Rostgaard K, Schmiegelow K, Soderhall S, Kolmannskog S, Vettenranta K, et al. Age- and sex-specific incidence of childhood leukemia by immunophenotype in the Nordic countries. *Journal of the National Cancer Institute*. 2003;95(20):1539-1544.
27. Bader JL, Miller RW. Neurofibromatosis and childhood leukemia. *Journal of Pediatrics*. 1978;92(6):925-929.
28. Hecht F, Hecht BK. Cancer in Ataxia-Telangiectasia patients. *Cancer Genetics and Cytogenetics*. 1990;46(1):9-19.
29. Howard SC, Metzger ML, Wilimas JA, Quintana Y, Pui CH, Robison LL, et al. Childhood cancer epidemiology in low-income countries. *Cancer*. 2008;112(3):461-472.
30. Greaves MF, Maia AT, Wiemels JL, Ford AM. Leukemia in twins: lessons in natural history. *Blood*. 2003;102(7):2321-2333.
31. Kadan-Lottick NS, Kawashima T, Tomlinson G, Friedman DL, Yasui Y, Mertens AC, et al. The risk of cancer in twins: A report from the Childhood Cancer Survivor Study. *Pediatric Blood & Cancer*. 2006;46(4):476-481.
32. Ford AM, Ridge SA, Cabrera ME, Mahmoud H, Steel CM, Chan LC, et al. Inutero rearrangements in the trithorax-related oncogene in infant leukemias. *Nature*. 1993;363(6427):358-360.
33. Wiemels JL, Cazzaniga G, Daniotti M, Eden OB, Addison GM, Masera G, et al. Prenatal origin of acute lymphoblastic leukaemia in children. *Lancet*. 1999;354(9189):1499-1503.
34. Gale KB, Ford AM, Repp R, Borkhardt A, Keller C, Eden OB, et al. Backtracking leukemia to birth: Identification of clonotypic gene fusion sequences in neonatal blood spots. *Proceedings of the National Academy of Sciences of the United States of America*. 1997;94(25):13950-13954.
35. McHale CM, Wiemels JL, Zhang LP, Ma XM, Buffler PA, Guo WH, et al. Prenatal origin of TEL-AMLI-positive acute lymphoblastic leukemia in children born in California. *Genes Chromosomes & Cancer*. 2003;37(1):36-43.

36. Hjalgrim LL, Madsen HO, Melbye M, Jorgensen P, Christiansen M, Andersen MT, et al. Presence of clone-specific markers at birth in children with acute lymphoblastic leukaemia. *British Journal of Cancer*. 2002;87(9):994-999.
37. Wiemels JL, Xiao ZJ, Buffler PA, Maia AT, Ma XM, Dicks BM, et al. In utero origin of t(8;21) AML1-ETO translocations in childhood acute myeloid leukemia. *Blood*. 2002;99(10):3801-3805.
38. Maia AT, van der Velden VHJ, Harrison CJ, Szczepanski T, Williams MD, Griffiths MJ, et al. Prenatal origin of hyperdiploid acute lymphoblastic leukemia in identical twins. *Leukemia*. 2003;17(11):2202-2206.
39. Panzer-Grumayer ER, Fasching K, Panzer S, Hettinger K, Schmitt K, Stockler-Ipsiroglu S, et al. Nondisjunction of chromosomes leading to hyperdiploid childhood B-cell precursor acute lymphoblastic leukemia is an early event during leukemogenesis. *Blood*. 2002;100(1):347-349.
40. Yagi T, Hibi S, Tabata Y, Morimoto A, Imashuku S. Detection of clonotypic IGH and TCR rearrangements in the neonatal blood spots of infants and children with B-cell precursor acute lymphoblastic leukemia. *Blood*. 2000;96(11):1358.
41. Taub JW, Konrad MA, Ge YB, Naber JM, Scott JS, Matherly LH, et al. High frequency of leukemic clones in newborn screening blood samples of children with B-precursor acute lymphoblastic leukemia. *Blood*. 2002;99(8):2992-2996.
42. Fasching K, Panzer S, Haas OA, Marschalek R, Gadner H, Panzer-Grumayer ER. Presence of clone-specific antigen receptor gene rearrangements at birth indicates an in utero origin of diverse types of early childhood acute lymphoblastic leukemia. *Blood*. 2000;95(8):2722-2724.
43. Gruhn B, Taub JW, Ge Y, Beck JF, Zell R, Hafer R, et al. Prenatal origin of childhood acute lymphoblastic leukemia, association with birth weight and hyperdiploidy. *Leukemia*. 2008;22(9):1692-1697.
44. Taub JW, Ge YB. The prenatal origin of childhood acute lymphoblastic leukemia. *Leukemia & Lymphoma*. 2004;45(1):19-25.
45. Mori H, Colman SM, Xiao ZJ, Ford AM, Healy LE, Donaldson C, et al. Chromosome translocations and covert leukemic clones are generated during normal fetal development. *Proceedings of the National Academy of Sciences of the United States of America*. 2002;99(12):8242-8247.
46. Olsen M, Madsen HO, Hjalgrim H, Gregers J, Rostgaard K, Schmiegelow K. Preleukemic TEL-AML1-positive clones at cell level of 10(-3) to 10(-4) do not persist into adulthood. *Journal of Pediatric Hematology Oncology*. 2006;28(11):734-740.
47. Maia AT, Ford AM, Jalali GR, Harrison CJ, Taylor GM, Eden OB, et al. Molecular tracking of leukemogenesis in a triplet pregnancy. *Blood*. 2001;98(2):478-482.
48. Tsuzuki S, Seto M, Greaves M, Enver T. Modeling first-hit functions of the t(12;21) TEL-AML1 translocation in mice. *Proceedings of the National Academy of Sciences of the United States of America*. 2004;101(22):8443-8448.
49. Morrow M, Horton S, Kioussis D, Brady HJM, Williams O. TEL-AML1 promotes development of specific hematopoietic lineages consistent with preleukemic activity. *Blood*. 2004;103(10):3890-3896.
50. Poole C, Greenland S, Luetters C, Kelsey JL, Mezei G. Socioeconomic status and childhood leukaemia: a review. *International Journal of Epidemiology*. 2006;35(2):370-384.

51. Ross JA, Potter JD, Reaman GH, Pendergrass TW, Robison LL. Maternal exposure to potential inhibitors of DNA topoisomerase II and infant leukemia (United States): A report from the children's cancer group. *Cancer Causes & Control*. 1996;7(6):581-590.
52. Strick R, Strissel PL, Borgers S, Smith SL, Rowley JD. Dietary bioflavonoids induce cleavage in the MLL gene and may contribute to infant leukemia. *Proceedings of the National Academy of Sciences of the United States of America*. 2000;97(9):4790-4795.
53. Preston DL, Kusumi S, Tomonaga M, Izumi S, Ron E, Kuramoto A, et al. Cancer incidence in atomic-bomb survivors .3. leukemia, lymphoma and multiple-myeloma, 1950-1987. *Radiation Research*. 1994;137(2):S68-S97.
54. Brain JD, Kavet R, McCormick DL, Poole C, Silverman LB, Smith TJ, et al. Childhood leukemia: Electric and magnetic fields as possible risk factors. *Environmental Health Perspectives*. 2003;111(7):962-970.
55. Greaves M. Infection, immune responses and the aetiology of childhood leukaemia. *Nature Reviews Cancer*. 2006;6(3):193-203.
56. Vasconcelos GM, Kang M, Pombo-de-Oliveira MS, Schiffman JD, Lorey F, Buffler P, et al. Adenovirus detection in Guthrie cards from paediatric leukaemia cases and controls. *British Journal of Cancer*. 2008;99(10):1668-1672.
57. Honkaniemi E, Talekar G, Huang W, Bogdanovic G, Forestier E, von Doblen U, et al. Adenovirus DNA in Guthrie cards from children who develop acute lymphoblastic leukaemia (ALL). *British Journal of Cancer*. 2010;102(5):796-798.
58. Kinlen L. Evidence for an infective cause of childhood leukemia - comparison of a scottish new town with nuclear reprocessing sites in britain. *Lancet*. 1988;2(8624):1323-1327.
59. Greaves MF. Speculations on the cause of childhood acute lymphoblastic-leukemia. *Leukemia*. 1988;2(2):120-125.
60. Vrooman LM, Silverman LB. Childhood acute lymphoblastic leukemia: update on prognostic factors. *Current Opinion in Pediatrics*. 2009;21(1):1-8.
61. Nordgren A, Heyman M, Sahlen S, Schoumans J, Soderhall S, Nordenskjold M, et al. Spectral karyotyping and interphase FISH reveal abnormalities not detected by conventional G-banding. Implications for treatment stratification of childhood acute lymphoblastic leukaemia: detailed analysis of 70 cases. *Eur J Haematol*. 2002;68(1):31-41.
62. Nordgren A. Hidden aberrations diagnosed by interphase fluorescence in situ hybridisation and spectral karyotyping in childhood acute lymphoblastic leukaemia. *Leukemia & Lymphoma*. 2003;44(12):2039-2053.
63. Kuchinskaya E, Heyman M, Nordgren A, Schoumans J, Staaf J, Borg A, et al. Array-CGH reveals hidden gene dose changes in children with acute lymphoblastic leukaemia and a normal or failed karyotype by G-banding. *British Journal of Haematology*. 2008;140(5):572-577.
64. Nachman JB, Heerema NA, Sather H, Camitta B, Forestier E, Harrison CJ, et al. Outcome of treatment in children with hypodiploid acute lymphoblastic leukemia. *Blood*. 2007;110(4):1112-1115.
65. Raimondi SC, Zhou YM, Mathew S, Shurtleff SA, Sandlund JT, Rivera GK, et al. Reassessment of the prognostic significance of hypodiploidy in pediatric patients with acute lymphoblastic leukemia. *Cancer*. 2003;98(12):2715-2722.

66. Wiemels JL, Greaves M. Structure and possible mechanisms of TEL-AML1 gene fusions in childhood acute lymphoblastic leukemia. *Cancer Research*. 1999;59(16):4075-4082.
67. Biondi A, Masera G. Molecular pathogenesis of childhood acute lymphoblastic leukemia. *Haematologica*. 1998;83(7):651-659.
68. Johansson B, Mertens F, Mitelman F. Clinical and biological importance of cytogenetic abnormalities in childhood and adult acute lymphoblastic leukemia. *Annals of Medicine*. 2004;36(7):492-503.
69. Forestier E, Andersen MK, Autio K, Blennow E, Borgstrom G, Golovleva I, et al. Cytogenetic patterns in ETV6/RUNXI-positive pediatric B-cell precursor acute lymphoblastic leukemia: A Nordic series of 245 cases and review of the literature. *Genes Chromosomes & Cancer*. 2007;46(5):440-450.
70. Romana SP, Poirel H, Leconiat M, Flexor MA, Jonveaux MMP, Macintyre EA, et al. High-frequency of t(12-21) in childhood b-lineage acute lymphoblastic-leukemia. *Blood*. 1995;86(11):4263-4269.
71. Forestier E, Heyman M, Andersen MK, Autio K, Blennow E, Borgstrom G, et al. Outcome of ETV6/RUNX1-positive childhood acute lymphoblastic leukaemia in the NOPHO-ALL-1992 protocol: frequent late relapses but good overall survival. *British Journal of Haematology*. 2008;140(6):665-672.
72. Zuna J, Ford AM, Peham M, Patel N, Saha V, Eckert C, et al. TEL deletion analysis supports a novel view of relapse in childhood acute lymphoblastic leukemia. *Clinical Cancer Research*. 2004;10(16):5355-5360.
73. Wood LD, Irvin BJ, Nucifora G, Luce KS, Hiebert SW. Small ubiquitin-like modifier conjugation regulates nuclear export of TEL, a putative tumor suppressor. *Proceedings of the National Academy of Sciences of the United States of America*. 2003;100(6):3257-3262.
74. Michaud J, Scott HS, Escher R. AML1 interconnected pathways of leukemogenesis. *Cancer Investigation*. 2003;21(1):105-136.
75. Song WJ, Sullivan MG, Legare RD, Hutchings S, Tan XL, Kufrin D, et al. Haploinsufficiency of CBFA2 causes familial thrombocytopenia with propensity to develop acute myelogenous leukaemia. *Nature Genetics*. 1999;23(2):166-175.
76. Pui C, Relling MV, Downing JR. Mechanisms of disease: Acute lymphoblastic leukemia. *New England Journal of Medicine*. 2004;350(15):1535-1548.
77. Rubnitz JE, Look AT. Molecular genetics of childhood leukemias. *Journal of Pediatric Hematology Oncology*. 1998;20(1):1-11.
78. Advani AS, Pendergast AM. Bcr-Abl variants: biological and clinical aspects. *Leukemia Research*. 2002;26(8):713-720.
79. Arica M, Schrappe M, Hunger S, Carroll WL, Conter V, Di Lorenzo P, et al. Clinical Outcome of 640 Children with Newly Diagnosed Philadelphia Chromosome-Positive Acute Lymphoblastic Leukemia Treated Between 1995 and 2005. *Blood*. 2008;112(11):213-214.
80. Rieder H, Schnittger S, Bodenstein H, Schwonzen M, Wormann B, Berkovic D, et al. Dic(9-20) - A new recurrent chromosome abnormality in adult acute lymphoblastic-leukemia. *Genes Chromosomes & Cancer*. 1995;13(1):54-61.
81. Slater R, Smit E, Kroes W, Bellomo MJ, Muhlematter D, Harbott J, et al. A nonrandom chromosome abnormality found in precursor-B lineage acute lymphoblastic-leukemia - Dic(9-20)(p13-q11). *Leukemia*. 1995;9(10):1613-1619.

82. Mitelman F, Johansson B, Mertens F. Mitelman Database of Chromosome Aberrations in Cancer. In; 2007.
83. Clark R, Byatt SA, Bennett CF, Brama M, Martineau M, Moorman AV, et al. Monosomy 20 as a pointer to dicentric (9;20) in acute lymphoblastic leukemia. *Leukemia*. 2000;14(2):241-246.
84. Heerema NA, Maben KD, Bernstein J, Breitfeld PP, Neiman RS, Vance GH. Dicentric (9;20)(p11;q11) identified by fluorescence in situ hybridization in four pediatric acute lymphoblastic leukemia patients. *Cancer Genetics and Cytogenetics*. 1996;92(2):111-115.
85. Gomez-Lazaro M, Fernandez-Gomez FJ, Jordan J. p53: Twenty five years understanding the mechanism of genome protection. *Journal of Physiology and Biochemistry*. 2004;60(4):287-307.
86. Spandidos DA, Liloglou T, Field JK. Prognostic-significance of oncogenes and tumor-suppressor genes in human malignancy. *Stem Cells*. 1993;11(3):194-198.
87. Li L, Zhang R, Fang ZY, Chen JN, Zhu ZL. Suppression of Vascular Endothelial Growth Factor (VEGF) Expression by Targeting the Bcr-Abl Oncogene and Protein Tyrosine Kinase Activity in Bcr-Abl-positive Leukaemia Cells. *Journal of International Medical Research*. 2009;37(2):426-437.
88. White MK, McCubrey JA. Suppression of apoptosis: role in cell growth and neoplasia. *Leukemia*. 2001;15(7):1011-1021.
89. Di Cristofano A, Pandolfi PP, Lf. The multiple roles of PTEN in tumor suppression. *Cell*. 2000;100(4):387-390.
90. McCubrey JA, Steelman LS, Abrams SL, Lee JT, Chang FM, Bertrand FE, et al. Roles of the RAF/MEK/ERK and PI3K/PTEN/AKT pathways in malignant transformation and drug resistance. In: *Advances in Enzyme Regulation, Vol 46, Proceedings*. Amsterdam: Elsevier Science Bv; 2006. p. 249-279.
91. Cantley LC, Neel BG, Bp. New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase AKT pathway. *Proceedings of the National Academy of Sciences of the United States of America*. 1999;96(8):4240-4245.
92. Aggerholm A, Gronbaek K, Guldberg P, Hokland P, Rk. Mutational analysis of the tumour suppressor gene MMAC1/PTEN in malignant myeloid disorders. *European Journal of Haematology*. 2000;65(2):109-113.
93. Bedolla R, Prihoda TJ, Kreisberg JI, Malik SN, Krishnegowda NK, Troyer DA, et al. Determining risk of biochemical recurrence in prostate cancer by immunohistochemical detection of PTEN expression end Akt activation. *Clinical Cancer Research*. 2007;13(13):3860-3867.
94. Dahia PLM, Aguiar RCT, Alberta J, Kum JB, Caron S, Sill H, et al. PTEN is inversely correlated with the cell survival factor Akt/PKB and is inactivated via multiple mechanisms in haematological malignancies. *Human Molecular Genetics*. 1999;8(2):185-193.
95. Perren A, Weng LP, Boag AH, Ziebold U, Thakore K, Dahia PLM, et al. Immunohistochemical evidence of loss of PTEN expression in primary ductal adenocarcinomas of the breast. *American Journal of Pathology*. 1999;155(4):1253-1260.
96. Roman-Gomez J, Jimenez-Velasco A, Castillejo JA, Agirre X, Barrios M, Navarro G, et al. Promoter hypermethylation of cancer-related genes: a strong

independent prognostic factor in acute lymphoblastic leukemia. *Blood*. 2004;104(8):2492-2498.
97. Stahl JM, Cheung M, Sharma A, Trivedi NR, Shanmugam S, Robertson GP, et al. Loss of PTEN promotes tumor development in malignant melanoma. *Cancer Research*. 2003;63(11):2881-2890.
98. Yang L, Kuang LG, Zheng HC, Li JY, Wu DY, Zhang SM, et al. PTEN encoding product: a marker for tumorigenesis and progression of gastric carcinoma. *World Journal of Gastroenterology*. 2003;9(1):35-39.
99. Liaw D, Marsh DJ, Li J, Dahia PLM, Wang SI, Zheng ZM, et al. Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. *Nature Genetics*. 1997;16(1):64-67.
100. Marsh DJ, Dahia PLM, Zheng ZM, Liaw D, Parsons R, Gorlin RJ, et al. Germline mutations in PTEN are present in Bannayan-Zonana syndrome. *Nature Genetics*. 1997;16(4):333-334.
101. Kumar R, Vaid VK, Kalra SK, Wf. Lhermitte-Duclos disease. *Childs Nervous System*. 2007;23(7):729-732.
102. Panigrahi AR, Pinder SE, Chan SY, Paish EC, Robertson JFR, Ellis IO. The role of PTEN and its signalling pathways, including AKT, in breast cancer; an assessment of relationships with other prognostic factors and with outcome. *Journal of Pathology*. 2004;204(1):93-100.
103. Yilmaz OH, Valdez R, Theisen BK, Guo W, Ferguson DO, Wu H, et al. Pten dependence distinguishes haematopoietic stem cells from leukaemia-initiating cells. *Nature*. 2006;441(7092):475-482.
104. Schondorf T, Gohring UJ, Roth G, Middel I, Becker M, Moser N, et al. Time to progression is dependent on the expression of the tumour suppressor PTEN in ovarian cancer patients. *European Journal of Clinical Investigation*. 2003;33(3):256-260.
105. Trotman LC, Wang XJ, Alimonti A, Chen ZB, Teruya-Feldstein J, Yang HJ, et al. Ubiquitination regulates PTEN nuclear import and tumor suppression. *Cell*. 2007;128(1):141-156.
106. Avellino R, Romano S, Parasole R, Bisogni R, Lamberti A, Poggi V, et al. Rapamycin stimulates apoptosis of childhood acute lymphoblastic leukemia cells. *Blood*. 2005;106(4):1400-1406.
107. Chong ZZ, Maiese K, Ez. The Src homology 2 domain tyrosine phosphatases SHP-1 and SHP-2: diversified control of cell growth, inflammation, and injury. *Histology and Histopathology*. 2007;22(11):1251-1267.
108. Wu CY, Sun MZ, Liu LJ, Zhou GW, Tm. The function of the protein tyrosine phosphatase SHP-1 in cancer. *Gene*. 2003;306:1-12.
109. Roskoski R. Signaling by Kit protein-tyrosine kinase - The stem cell factor receptor. *Biochemical and Biophysical Research Communications*. 2005;337(1):1-13.
110. Koyama M, Oka T, Ouchida M, Nakatani Y, Nishiuchi R, Yoshino T, et al. Activated proliferation of B-cell lymphomas/leukemias with the SHP1 gene silencing by aberrant CpG methylation. *Laboratory Investigation*. 2003;83(12):1849-1858.
111. Oka T, Ouchida M, Koyama M, Ogama Y, Takada S, Nakatani Y, et al. Gene silencing of the tyrosine phosphatase SHP1 gene by aberrant methylation in leukemias/lymphomas. *Cancer Research*. 2002;62(22):6390-6394.
112. Reddy J, Shivapurkar N, Takahashi T, Parikh G, Stastny V, Echebiri C, et al. Differential methylation of genes that regulate cytokine signaling in lymphoid and hematopoietic tumors. *Oncogene*. 2005;24(4):732-736.

113. Mena-Duran AV, Togo SH, Bazhenova L, Cervera J, Bethel K, Senent ML, et al. SHP1 expression in bone marrow biopsies of myelodysplastic syndrome patients: a new prognostic factor. *British Journal of Haematology*. 2005;129(6):791-794.
114. Amin HM, Hoshino K, Yang H, Lin Q, La R, Garcia-Manero G, et al. Decreased expression level of SH2 domain-containing protein tyrosine phosphatase-I (ShpI) is associated with progression of chronic myeloid leukaemia. *Journal of Pathology*. 2007;212(4):402-410.
115. Lu YL, Yu QH, Liu JH, Zhang JY, Wang HW, Koul D, et al. Src family protein-tyrosine kinases alter the function of PTEN to regulate phosphatidylinositol 3-kinase/AKT cascades. *Journal of Biological Chemistry*. 2003;278(41):40057-40066.
116. Min YH, Cheong JW, Lee MH, Kim JY, Lee ST, Hahn JS, et al. Elevated S-phase kinase-associated protein 2 protein expression in acute myelogenous leukemia: Its association with constitutive phosphorylation of phosphatase and tensin homologue protein and poor prognosis. *Clinical Cancer Research*. 2004;10(15):5123-5130.
117. Martelli AM, Nyakern M, Tabellini G, Bortul R, Tazzari PL, Evangelisti C, et al. Phosphoinositide 3-kinase/Akt signaling pathway and its therapeutical implications for human acute myeloid leukemia. *Leukemia*. 2006;20(6):911-928.
118. Cheong JW, Eom JI, Maeng HY, Lee ST, Hahn JS, Ko YW, et al. Phosphatase and tensin homologue phosphorylation in the C-terminal regulatory domain is frequently observed in acute myeloid leukaemia and associated with poor clinical outcome. *British Journal of Haematology*. 2003;122(3):454-456.
119. Skolnik JM, Adamson PC. Tyrosine kinase inhibitors in pediatric malignancies. *Cancer Investigation*. 2007;25(7):606-612.
120. Poyer F, Coquerel B, Pegahi R, Cazin L, Norris V, Vannier JP, et al. Secretion of MMP-2 and MMP-9 induced by VEGF autocrine loop correlates with clinical features in childhood acute lymphoblastic leukemia. *Leukemia Research*. 2009;33(3):407-417.
121. Cross MJ, Dixelius J, Matsumoto T, Claesson-Welsh L. VEGF-receptor signal transduction. *Trends in Biochemical Sciences*. 2003;28(9):488-494.
122. Podar K, Anderson KC. The pathophysiologic role of VEGF in hematologic malignancies: therapeutic implications. *Blood*. 2005;105(4):1383-1395.
123. Santos SCR, Dias S. Internal and external autocrine VEGF/KDR loops regulate survival of subsets of acute leukemia through distinct signaling pathways. *Blood*. 2004;103(10):3883-3889.
124. Li WW, Hutnik M, Gehr G. Antiangiogenesis in haematological malignancies. *British Journal of Haematology*. 2008;143(5):622-631.
125. Kessler T, Fehrmann F, Bieker R, Berdel WE, Mesters RM. Vascular endothelial growth factor and its receptor as drug targets in hematological malignancies. *Current Drug Targets*. 2007;8(2):257-268.
126. Aguayo A, Giles F, Albitar M. Vascularity, angiogenesis and angiogenic factors in leukemias and myelodysplastic syndromes. *Leukemia & Lymphoma*. 2003;44(2):213-222.
127. de Bont E, Rosati S, Jacobs S, Kamps WA, Vellenga E. Increased bone marrow vascularization in patients with acute myeloid leukaemia: a possible role for vascular endothelial growth factor. *British Journal of Haematology*. 2001;113(2):296-304.

128. Matuszewski L, Persigehl T, Wall A, Meier N, Bieker R, Kooijman H, et al. Assessment of bone marrow angiogenesis in patients with acute myeloid leukemia by using contrast-enhanced MR imaging with clinically approved iron oxides: Initial experience. *Radiology*. 2007;242(1):217-224.
129. Zhang YY, Pillai G, Gatter K, Blazquez C, Pezzella F, Watt SM. Expression and cellular localization of vascular endothelial growth factor A and its receptors in acute and chronic leukemias: An immunohistochemical study. *Human Pathology*. 2005;36(7):797-805.
130. Negaard HFS, Iversen N, Bowitz-Lothe IM, Sandset PM, Steinsvik B, Ostenstad B, et al. Increased bone marrow microvascular density in haematological malignancies is associated with differential regulation of angiogenic factors. *Leukemia*. 2009;23(1):162-169.
131. Rajkumar SV, Mesa RA, Tefferi A. A review of angiogenesis and anti-angiogenic therapy in hematologic malignancies. *Journal of Hematotherapy & Stem Cell Research*. 2002;11(1):33-47.
132. Avramis IA, Panosyan EH, Dorey F, Holcenberg JS, Avramis VI. Correlation between high vascular endothelial growth factor-A serum levels and treatment outcome in patients with standard-risk acute lymphoblastic leukemia: A report from Children's Oncology Group study CCG-1962. *Clinical Cancer Research*. 2006;12(23):6978-6984.
133. Yetgin S, Yenicesu I, Cetin M, Tuncer M. Clinical importance of serum vascular endothelial and basic fibroblast growth factors in children with acute lymphoblastic leukemia. *Leukemia & Lymphoma*. 2001;42(1-2):83-88.
134. Koomagi R, Zintl F, Sauerbrey A, Volm M. Vascular endothelial growth factor in newly diagnosed and recurrent childhood acute lymphoblastic leukemia as measured by real-time quantitative polymerase chain reaction. *Clinical Cancer Research*. 2001;7(11):3381-3384.
135. Pinkel D, Straume T, Gray JW. Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. *Proceedings of the National Academy of Sciences of the United States of America*. 1986;83(9):2934-2938.
136. Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. *Genome Research*. 1996;6(10):986-994.
137. Forestier E, Gauffin F, Andersen MK, Autio K, Borgstrom G, Golovleva I, et al. Clinical and cytogenetic features of pediatric dic(9;20)(p13.2;q11.2)-positive B-Cell precursor acute lymphoblastic leukemias: A nordic series of 24 cases and review of the literature. *Genes Chromosomes & Cancer*. 2008;47(2):149-158.
138. Schoumans J, Johansson B, Corcoran M, Kuchinskaya E, Golovleva I, Grander D, et al. Characterisation of dic(9;20)(p11-13;q11) in childhood B-cell precursor acute lymphoblastic leukaemia by tiling resolution array-based comparative genomic hybridisation reveals clustered breakpoints at 9p13.2 and 20q11.2. *British Journal of Haematology*. 2006;135(4):492-499.
139. Pichler H, Moricke A, Mann G, Teigler-Schlegel A, Niggli F, Nebral K, et al. Prognostic relevance of dic(9;20)(p11;q13) in childhood B-cell precursor acute lymphoblastic leukaemia treated with Berlin-Frankfurt-Munster (BFM) protocols containing an intensive induction and post-induction consolidation therapy. *British Journal of Haematology*. 2010;149(1):93-100.
140. Lonnerholm G, Nordgren A, Frost BM, Jonsson OG, Kanerva J, Nygaard R, et al. Dic(9;20)(p13;q11) in childhood acute lymphoblastic leukaemia is related to low cellular resistance to asparaginase, cytarabine and corticosteroids. *Leukemia*. 2009;23(1):209-212.

141. Johansson B, Mertens F, Mitelman F. Primary vs, secondary neoplasia-associated chromosomal abnormalities - Balanced rearrangements vs, genomic imbalances? *Genes Chromosomes & Cancer*. 1996;16(3):155-163.
142. Mitelman F, Johansson B, Mertens F. The impact of translocations and gene fusions on cancer causation. *Nature Reviews Cancer*. 2007;7(4):233-245.
143. Strefford JC, Worley H, Barber K, Moorman AV, Harrison CJ. Genome complexity in patients with acute lymphoblastic leukaemia revealed by array-based comparative genomic hybridization. *Blood*. 2006;108(11):584A-584A.
144. Haak PT, Busik JV, Kort EJ, Tikhonenko M, Paneth N, Resau JH. Archived Unfrozen Neonatal Blood Spots Are Amenable to Quantitative Gene Expression Analysis. *Neonatology*. 2009;95(3):210-216.
145. Sjoholm MIL, Dillner J, Carlson J. Assessing quality and functionality of DNA from fresh and archival dried blood spots and recommendations for quality control guidelines. *Clinical Chemistry*. 2007;53(8):1401-1407.
146. Skinner JR, Chong B, Fawkner M, Webster DR, Hegde M. Use of the newborn screening card to define cause of death in a 12-year-old diagnosed with epilepsy. *Journal of Paediatrics and Child Health*. 2004;40(11):651-653.
147. Lausten-Thomsen U, Madsen HO, Vestergaard TR, Hjalgrim H, Lando A, Schmiegelow K. Increased risk of ALL among premature infants is not explained by increased prevalence of pre-leukemic cell clones. *Blood Cells Molecules and Diseases*. 2010;44(3):188-190.
148. Beillard E, Pallisgaard N, van der Velden VHJ, Bi W, Dee R, van der Schoot E, et al. Evaluation of candidate control genes for diagnosis and residual disease detection in leukemic patients using 'real-time' quantitative reverse-transcriptase polymerase chain reaction (RQ-PCR) - a Europe against cancer program. *Leukemia*. 2003;17(12):2474-2486.
149. Gabert J, Beillard E, van der Velden VHJ, Bi W, Grimwade D, Pallisgaard N, et al. Standardization and quality control studies of 'real-time' quantitative reverse transcriptase polymerase chain reaction of fusion gene transcripts for residual disease detection in leukemia - A Europe Against Cancer Program. *Leukemia*. 2003;17(12):2318-2357.
150. Hong DL, Gupta R, Ancliff P, Atzberger A, Brown J, Soneji S, et al. Initiating and cancer-propagating cells in TEL-AML1-associated childhood leukemia. *Science*. 2008;319(5861):336-339.
151. Wertheim JA, Miller JP, Xu LW, He YP, Pear WS. The biology of chronic myelogenous leukemia: mouse models and cell adhesion. *Oncogene*. 2002;21(56):8612-8628.
152. Yang J, Liu J, Zheng J, Du W, He Y, Liu W, et al. A reappraisal by quantitative flow cytometry analysis of PTEN expression in acute leukemia. *Leukemia*. 2007;21(9):2072-2074.