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Identification and Molecular Characterization of Fusion Genes in Hematological Malignancies and Disorders

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Summary

This project consisted of two distinct parts. First, a patient who suffered from and died of AML exhibited a rather unusual constellation of translocations. In a previous project the ETV6 locus, coding for a hematopoietic transcription factor, was shown to be affected by the translocation. The elucidation of the fusion partner of ETV6 and the characterization of the resulting fusion transcript were the goal of this project.
With the help of RACE PCR, cloning, and sequencing the neurotrophic receptor kinase NTRK3 was identified as partner. FISH assays proved the existence of the underlying chromosomal translocation. Further molecular analyses unveiled the structural organization of the chimeric transcript. Additionally, alternative splicing of the NTRK3 RNA was detected. The discovery of this exact fusion transcript turned out to be unique as it has not been previously described for AML.

The second part of the project dealt with the identification of fusion genes again, though the intended method had to be established first.
Two patients with eosinophilia constituted unusual translocations that have only been defined by conventional cytogenetic methods. The chromosomal breakpoints had to be specified with the help of bacterial artificial chromosomes (BACs).
In a first and very time consuming step the obtainment of the import license for *E.coli* harboring BACs issued by the Ministry of Health, Family, and Youth was necessary to actually start working on this part of the project. The adequate BACs were searched for in different data bases and were then ordered over the internet at BACPAC Resources Center at Children's Hospital Oakland Research Institute (CHORI) in California, USA. A suitable method for labeling BAC DNA had to be found in the next step. Finally, the most convenient way for hybridization of the self-labeled probes was developed so that this procedure could easily be adopted for routine cytogenetics if required.
Fluorescently labeled BACs were then used to localize the breakpoints and to find genes affected by the translocation. In one case, a particular chromosomal region was tested for the existence of the breakpoint by covering up the area with different BAC probes. Unfortunately, no breakpoint could be located to the assumed chromosome band, indicating that conventional cytogenetics can be very tricky and sometimes additional analyses using appropriate probes may be required for exact evaluation of the translocation.

In the other case, self-labeled BAC probes were used to walk along a target chromosome arm until the breakpoint could be narrowed down to a smaller region. Additional BAC hybridization assays were used to identify the candidate gene, lipid phosphatase TPIP/TPTE2, which is most likely to be involved in the translocation.

Zusammenfassung

Dieses Projekt setzte sich aus zwei Teilen zusammen. Ein Teil beschäftigte sich mit dem Fall eines AML Patienten, der eine recht ungewöhnliche Konstellation an Translokationen aufwies. In einem vorangegangen Projekt wurde bereits gezeigt, dass der ETV6 Locus, der für einen hämatopoetischen Transkriptionsfaktor kodiert, von einer Translokation betroffen ist. Das Ziel dieses Teilprojekts war es, den Fusionspartner von ETV6 zu ermitteln und das resultierende Fusionstranskript zu charakterisieren.
Mit Hilfe von RACE PCR, Klonierungen und Sequenzanalysen wurde die neurotrophe Rezeptorkinase NTRK3 als Fusionspartner identifiziert. Fluoreszenz in-situ Hybridisierungen konnten schließlich die zugrunde liegende chromosomale Translokation beweisen. Durch weitere molekulare Analysen konnte zum einen der strukturelle Aufbau des chimären Transkripts beschrieben und zum anderen alternatives Spleißen der NTRK3-RNA festgestellt werden. Die Entdeckung genau dieses Fusionstranskripts stellte sich als einzigartig heraus, da es in dieser Form bei AML in der Literatur bisher noch nicht beschrieben wurde.

Der zweite Teil des Projekts beschäftigte sich ebenfalls mit der Identifizierung von Fusionsgenen, jedoch musste die dafür vorgesehene Methode erst etabliert werden.
Zwei Patienten mit Eosinophilie wiesen ungewöhnliche Translokationen auf, die routinemäßig nur mittels konventioneller Zytogenetik definiert wurden. Die chromosomalen Bruchpunkte sollten mit Hilfe von BACs weiter spezifiziert werden.
Der erste und besonders zeitaufwändige Schritt bestand darin, die Einfuhrbewilligung von BACs zu erhalten, die durch das Bundesministerium für Gesundheit, Familie und Jugend ausgestellt wurde. Erst danach konnten die passenden BACs aus diversen öffentlich zugänglichen Internet-Datenbanken ausgesucht und über die Website von BACPAC Resources Center at Children's Hospital Oakland Research Institute (CHORI) in Kalifornien bestellt werden. Weiters wurde eine adäquate Methode zur Herstellung von fluoreszenzmarkierten BAC-Sonden etabliert und die komfortabelste Arbeitsweise für Hybridisierungen gefunden, damit die Methode bei Bedarf einfacher für Routinearbeiten übernommen werden kann.
Die fluoreszenzmarkierten BACs wurden schließlich verwendet, um Bruchpunkte zu lokalisieren und um Gene zu finden, die von der Translokation betroffen sind.
In einem der beiden Patientenfälle wurde eine bestimmte chromosomale Region auf einen Bruchpunkt untersucht, indem dieser Bereich durch BAC-Sonden abgedeckt

wurde. Jedoch konnte kein Bruchpunkt in der angenommenen Chromsomenbande lokalisiert werden. Dieses Beispiel zeigte, dass konventionelle Zytogenetik sehr kompliziert sein kann und daher manchmal zusätzliche Analysen durch passende Sonden erforderlich sein können, um die Translokation genau zu definieren.
Im weiteren Patientenfall wurden die BAC-Sonden verwendet, um entlang eines betroffenen Chromosomenarms zu wandern. Der Bruchpunkt wurde so auf einen weitaus kleineren Bereich eingeengt und weitere BAC-Hybridisierungen identifizierten ein Kandidat-Gen, Lipidphosphatase TPIP/TPTE2, das mit sehr hoher Wahrscheinlichkeit von der Translokation betroffen ist.

Contents

1 Introduction

1.1 Hematopoiesis

Hematopoiesis, from Ancient Greek *haima* meaning blood and *poiesis* meaning production [1], is the biological process by which the blood cells of an organism are formed. It is the mechanism that guarantees that the predominantly short lived blood cells are produced throughout life by means of hematopoietic stem cells (HSC) [2]. As with all stem cells, HSCs are capable of both self-renewal and differentiation [3]. Therefore, HSCs are able to produce additional HSCs (self-renewal) and give rise to all blood cell lineages (differentiation).

HSCs normally reside in small numbers in the adult bone marrow where they are on top of a hierarchical tree of progenitor cells eventually differentiating into mature blood cells [2]. HSCs can be divided into three different populations depending on their maturation state: long-term self-renewing HSC (LT-HSC) giving rise to short-term self-renewing HSC (ST-HSC) which in turn give rise to multipotent progenitor (MPP) cells without self-renewal potential. Multipotent progenitor cells differentiate either into common lymphoid progenitors (CLP) or common myeloid progenitors (CMP). CLPs generate the lymphoid lineages consisting of B and T lymphocytes, as well as natural killer (NK) cells and dendritic cells (DC). CMPs are the precursors of the myeloid lineage differentiating into erythrocytes, platelets, macrophages, granulocytes, and dendritic cells [3]. Granulocytes are further divided into three classes according to their morphology and staining properties: neutrophils, basophils, and eosinophils [4]. An overview of hematopoiesis is given in figure 1.1.

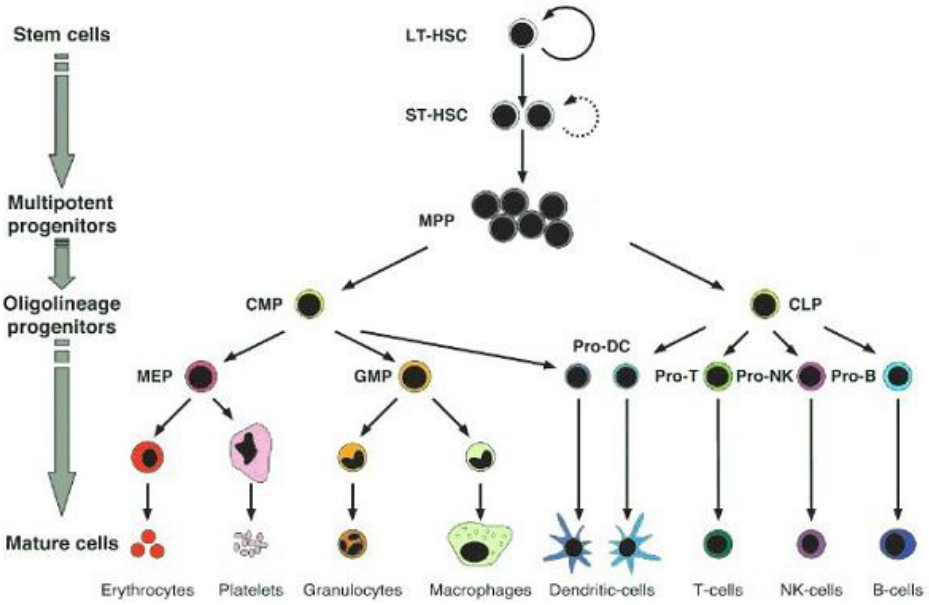

Figure 1.1 *Differentiation pathway of hematopoietic cells. MEP, megakaryocyte erythrocyte precursor; GMP, granulocyte macrophage precursor; NK, natural killer (picture from [5]).*

Hematopoiesis is a complex developmental process that involves various anatomical sites during embryogenesis including the yolk sac, the placenta and eventually the fetal liver. Postnatally, the bone marrow becomes the major site of hematopoiesis [6].

1.1.1 Regular and aberrant control of hematopoiesis

Both survival promoting (anti-apoptotic) and death inducing (pro-apoptotic) molecules are involved in the process of survival, proliferation and differentiation of all progenitor and blood cell types [7] in order to maintain a finely tuned steady-state level of HSCs in the bone marrow which constantly provide progenitors for the various hematopoietic lineages. The differentiation of these lineages is a stepwise process characterized by the tightly regulated expression of transcription factors, growth factors such as cytokines, and growth factor receptors whose alternating combination determines a cell's differentiation and maturation path [5]. The activity of these proteins must be very accurately regulated to maintain a homeostatic balance of these molecules. To date, more than two dozen 'hematopoietic transcription factors' exist of which nearly all were found to be associated with hematopoietic malignancies, i.e. leukemia. What makes the incidence of leukemia even more easily to occur is the fact that the transcription factors critical for hematopoiesis involve virtually all classes of DNA binding proteins, rather than just a particular family. The majority of the transcription factors contribute to

hematopoietic malignancies as chimeric proteins produced by chromosomal translocations. Target genes are therefore inappropriately activated or repressed resulting in aberrant survival and apoptosis regulation [2,5]. Not only mutated transcriptional regulators contribute to the development of hematopoietic disorders but also other proteins including mutated growth factor receptors, kinases and intracellular signaling molecules such as the GTP binding Ras proteins. These mutations also allow affected cells to bypass normal control of growth, differentiation, and apoptosis [8].

One particular transcription factor, ETV6, fundamental in adult hematopoiesis and its role in hematological malignancies are discussed in great detail below. Thereafter, two different forms of hematopoietic disorders will also be described as well: the pathogenic proliferation of myeloid progenitor cells resulting in AML, and the aberrant proliferation of eosinophilic cells resulting in eosinophilia.

1.2 ETV6 – a versatile transcription factor

The ETV6 (*ets*[1] variant gene 6) or TEL[2] gene on the short arm of chromosome 12 at band 12p13 encodes a transcriptional repressor of the *ets* transcription factor family [9]. It is a large family of transcriptional regulators with more than 45 members, which play important roles in a variety of cellular processes including proliferation, differentiation, migration/tissue remodeling, angiogenesis, apoptosis, as well as hematopoiesis and cell transformation [10,11]. Each *ets* family member possesses a highly conserved region of about 85 amino acids, the *ets* domain. It is a sequence specific DNA binding domain, which recognizes a purine rich GGAA/T motif in promoters and enhancers of various genes [9,10].

1.2.1 Structural organization and associated functional activities of the ETV6 protein

ETV6 is a 452 amino acid protein encoded by 8 exons that bears its *ets* domain at its carboxy terminus [12,13]. The *ets* domain harbors DNA binding properties but also

[1] E26-transforming specific

[2] Transformation ets leukemia

mediates protein-protein interactions [9]. As with one third of *ets* family members, ETV6 contains a helix-loop-helix (HLH) domain (also known as pointed or SAM[3] domain), which mediates homotypic oligomerization of ETV6 molecules and also heterotypic interactions with other proteins of the transcriptional regulation apparatus or of signaling cascades [9,10]. See figure 1.2 for ETV6 structure.

Figure 1.2 *Schematic representation of ETV6. The structural organization of ETV6 with its exon and domain boundaries is demonstrated (illustrated according to [13]).*

ETV6 activity as transcriptional repressor is achieved by at least two different domains: the HLH domain and the central region located between the SAM and *ets* domain [9,10] encoded by part of exon 4 and the entire exon 5. This central domain was shown to interact with corepressors like mSin3A/B or SMRT, which recruit histone deacetylases (HDAC), whereas the HLH domain represses gene transcription independently from corepressors [9,10,14]. Possibly, one of the two domains is preferentially active depending on the promoter or the physiologic state of the cell [14].

1.2.2 Expression profile and subcellular localization

In contrast to most *ets* proteins, ETV6 is widely expressed in normal tissues [9,10] and it is relatively abundant in hematopoietic cells [15]. Two distinct isoforms of the ETV6 protein exist that differ in their length by 42 amino acids due to an alternative start codon. Considering the major isoform that is pictured in figure 1.2 this alternative start codon lies within coding exon 2 and therefore both isoforms contain the HLH as well as the *ets* domain [12].

ETV6 is predominantly found as a nuclear phosphoprotein. It was shown that a stretch of about 20 amino acids in the DNA binding domain is necessary for localization within the nucleus. However, the underlying mechanism is not understood [16].

[3] Sterile alpha motif

1.2.3 ETV6, its functions and its dysfunctions

1.2.3.1 The physiological role of ETV6

As mentioned earlier, ETV6 is a transcriptional repressor that works either by itself or in combination with other proteins called corepressors. Wang et al. showed that its functionality is essential in the mouse embryo where it is required for angiogenesis in the yolk sac and that embryos homozygous for ETV6 loss (*ETV6*$^{-}$/$^{-}$) die during embryonic development. Additionally, they showed that ETV6 was not necessary for fetal liver hematopoiesis, though it is the first transcription factor specifically required for bone marrow hematopoiesis in early postnatal life [15]. In the bone marrow ETV6 is needed for the production of erythroid, myeloid, mast, and megakaryocytic cells. As far as the committed lymphoid lineage is concerned, ETV6 is not required for the proliferation and differentiation *per se* but it is needed for maintenance of a normal pool of lymphoid progenitors in the bone marrow [15].

Therefore, ETV6 plays an important role in the development and maintenance of HSCs and multipotent progenitors in the bone marrow niche. ETV6 is also said to probably be involved in adhesion-mediated cellular processes as *ETV6*$^{-}$/$^{-}$ HSCs or progenitors fail in stably colonizing the bone marrow [15].

Even though very little is known about the upstream regulators of ETV6 and its downstream targets, it is thought to function as a tumor suppressor. This idea is supported by its ability to inhibit growth of Ras transformed NIH3T3 fibroblasts and its ability to inhibit expression of a metalloproteinase that is elemental for tissue remodeling and tumor invasion [9,11]. Also, ETV6 is involved in a great number of translocations associated with leukemia. In such cases, one allele of the ETV6 locus is involved in a translocation while the other allele is often found to be deleted or inactivated. This, too, makes ETV6 a candidate tumor suppressor gene [10,17].

1.2.3.2 The leukemogenic role of ETV6

All the above features of ETV6 were elucidated only after it was originally identified as the fusion partner of the platelet-derived growth factor receptor β (PDGFRB) which is a protein tyrosine kinase (PTK). The underlying translocation t(5;12)(q33;p13) and the expression of the resulting fusion gene, ETV6-PDGFRB, were critical for the development of chronic myelomonocytic leukemia (CMML) [9,12].

ETV6 is located on band 12p13 which is genetically very unstable and fragile and thus susceptible to chromosomal rearrangements [17]. It is not very surprising that in the

last decade ETV6 was shown to be involved in a variety of translocations associated with hematological malignancies of both myeloid as well as lymphoid origin [9,18]. The diversity of ETV6 rearrangements is striking considering that the breakpoints do not cluster within a particular region of the gene [12]. For instance, in cases where ETV6 constitutes the 5´ part of the fusion gene, transcripts can comprise the first 4 exons or alternatively exons 1 through 5 [19,20]. On the other hand when ETV6 constitutes the 3´ part of the fusion, transcripts were shown to contain either exons 2 through 8 or exons 3 through 8 [21].
As shown in figure 1.3, more than 20 fusion partners to the ETV6 gene locus are known to date and most of them are either tyrosine kinases or transcription factors [9,17].

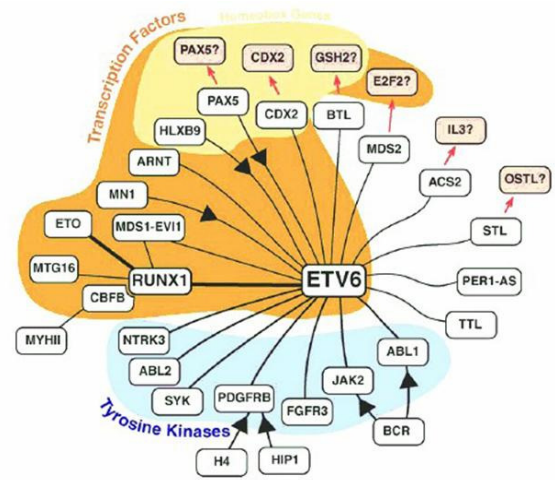

Figure 1.3 *ETV6 and several of its fusion partners associated with leukemia. Generally, ETV6 constitutes the 5´part of the fusion genes unless otherwise indicated by a black arrow head. Red arrows indicate transcriptional upregulation whereas question marks denote that upregulation or its relevance requires additional data. Transcription factors highlighted in yellow belong to the Homeobox gene family (figure from [9]).*

The role of ETV6 in hematological malignancies resulting from translocations is not entirely understood. However, a few mechanisms by which ETV6 contributes to the aberrant function of fusion products have been proposed. For instance, if fused to PTKs, the HLH domain of ETV6 serves as dimerization module which allows homodimerization of the fusion product. Consequently, the kinase domain of the PTK becomes constitutively activated which in turn leads to autophosphorylation of the fusion protein and of downstream signaling molecules [9].
Fusions between ETV6 and PTKs are found in a variety of hematological diseases, e.g. CMML, AML, acute lymphoid leukemia (ALL) or myelodysplastic syndrome (MDS). They

have also been described for solid tumors, like congenital fibrosarcoma or secretory breast carcinoma [9].

The transcription factor RUNX1 (AML1) was the second fusion partner of ETV6 to be identified [9]. AML1, a transcriptional activator, plays an essential role in adult hematopoiesis, like ETV6 itself [9,15].
The ETV6-AML1 fusion turned out to be the most common fusion gene in pediatric B-cell ALL where it is detected in about 17% to 25% of all cases [9,18].
It is not understood yet how this fusion disturbs the normal gene activation in hematopoiesis. However, data indicate that the chimeric protein still acts on AML1 target genes but it represses rather than activates transcription of target genes. Both the ETV6 HLH domain as well as the central domain is necessary for repression of AML1 targets [9].
In cases of aberrant MN1-ETV6, PAX5-ETV6 or HLXB9-ETV6 fusions, the *ets* domain of ETV6 seems to contribute to dysregulation via its DNA binding domain [9,22].

As can be seen from the above information on ETV6 and its fusion partners a lot of work remains to be done in order to elucidate the molecular mechanisms driving the oncological path of hematopoiesis.

1.3 Acute myeloid leukemia (AML)

AML is a clonal disease of myeloid precursors showing defective maturation and genetic abnormalities [23,24]. The failure in differentiation of myeloid stem cells results in the accumulation of wildly proliferating, non-functioning, immature cells called blasts [25,26]. In healthy persons, circulating blast cells are very rare and constitute about 0,1% of mononuclear cells [24].
Blast cells are easily distinguished form normal hematopoietic cells in blood or bone marrow samples because of their characteristic appearance. They measure 12 to 20 µm in diameter and have a high nucleocytoplasmic ratio with a round or slightly oval nucleus [24]. The cytoplasm is basophilic and appears pale blue [23,24]. The nucleus shows a fine, dense chromatin structure with several nucleoli [23]. Figure 1.4 brings out the difference between healthy bone marrow cells and malignant accumulation of blasts.

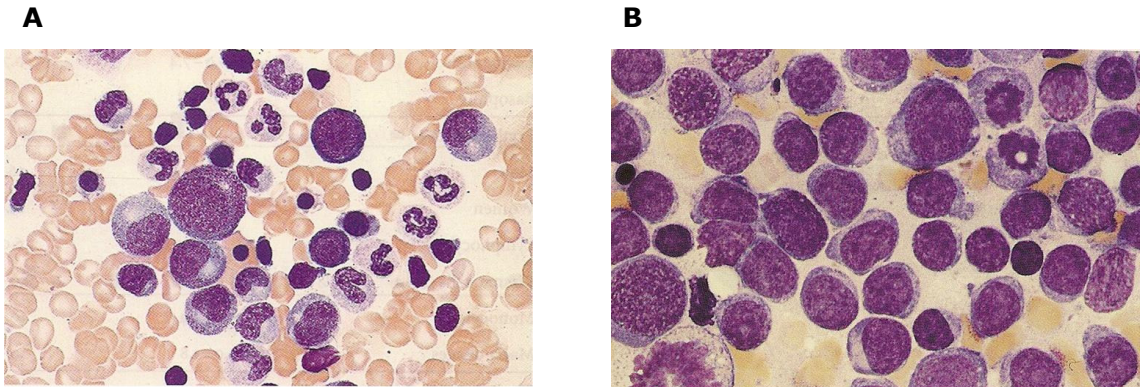

Figure 1.4 A, B *Healthy versus malignant bone marrow cells. Normal bone marrow smear is shown in A, whereas B demonstrates bone marrow cells from a patient suffering from the AML subtype M0 (pictures from [23]).*

1.3.1 Epidemiology and etiology

AML accounts for approximately 25% of all adult leukemia in the Western World and is therefore the most common type of leukemia in adults. Nevertheless, it has the lowest survival rate of all hematological malignancies. AML is a consistently fatal disease with a mortality rate higher for males than for females. The U.S. age-adjusted mortality rate is at 3,5 and 2,2 per 100.000 for males and females, respectively. [27].

In 2004, an Italian study with 1005 participating AML patients showed that median survival of patients age >60 years was only 5 to 7 months despite therapy.

Many risk factors contribute to the development of AML, including age, precedent hematological disease and genetic disorders, as well as exposure to radiation or chemotherapy. The latter being able to induce AML (secondary AML) in 10% to 20% of patients who were treated for other malignancies between 5 or 10 years earlier [27].

1.3.2 Classification of AML disease

Different manifestations of AML are categorized according to peripheral blood and bone marrow features. To date, the French-American-British (FAB) classification of AML is most generally accepted, albeit the World Health Organization (WHO) classification will be increasingly adopted. There is one very important difference between these two classifications. According to the FAB classification the bone marrow blast cell percentage must be at least 30%, whereas AML is diagnosed with 20% bone marrow blast cells according to the WHO classification [24].

Table 1.1 FAB classification of AML [23,24]

M0	AML with minimal myeloid differentiation	blasts of myeloid origin, <3% of blasts MPO negative
M1	AML without maturation	>90% blasts, >3% of blasts MPO positive, <10% mo, <10% gra
M2	AML with granulocytic maturation	30-89% blasts, >10% gra, <20% mo
M3	acute promyelocytic leukemia	hypergranular, Auer rods, <20% mo
M3v	hypogranular variant of M3	hypogranular, monocytoid nuclei, <20% mo
M4	acute myelomonocytic leukemia	>30% blasts, >20% gra, >20%mo
M4Eo	acute myelomonocytic leukemia with eosinophilia	>30% blasts, >20% gra, >20%mo, abnormal eosinophil count
M5a	acute monoblastic leukemia	>80% mo immature
M5b	acute monocytic leukemia	>80% mo mature
M6	erythroleukemia	>30% blasts, >50% ery
M7	acute megakaryoblastic leukemia	>30% blasts (megakaryoblasts, CD41/CD61 positive)

MPO, myeloperoxidase; gra, granulocytopoiesis; mo, monocytopoiesis; ery, erythropoiesis.

The WHO classification is somewhat differently organized and considers cytogenetic aberrations and gene rearrangements, most of which are chromosomal translocations resulting in fusion genes.

Table 1.2 shows the WHO classification of AML which was adopted from reference [23]. The latest version of the WHO classification of hematopoietic malignancies (WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues, New Fourth Edition) was released in 2008.

Table 1.2 Classification of AML according to the WHO (adopted from reference [23])

AML with recurrent cytogenetic abnormalities
- AML with t(8;21)(q22;q22): *AML1-ETO*
- AML with abnormal BM eosinophils inv(16)(p13q22): *CBFβ-MYH11*
- acute promyelocytic leukemia with t(15;17)(q22;q12): *PML-RARα*
- AML with 11q23 (*MLL*) abnormalities
AML with multilineage dysplasia
AML and myelodysplastic syndromes, therapy-related
AML not otherwise categorized
- AML minimally differentiated
- AML without maturation
- AML with maturation
- acute myelomonocytic leukemia
- acute monoblastic and monocytic leukemia
- acute erythroid leukemia
- acute megakaryoblastic leukemia
- acute basophilic leukemia
- acute panmyelosis with myelofibrosis
- myeloid sarcoma
Acute leukemia of ambiguous lineage
- undifferentiated acute leukemia
- bilineal acute leukemia
- biphenotypic acute leukemia

BM, bone marrow.

1.3.3 Chromosomal aberrations as prognostic factors

Considering the above information it becomes clear that AML is a very heterogeneous disease with regard to clinical features and genetic defects. Acquired genetic defects do not only provide a basis for development of AML, they are also very important prognostic factors for therapy outcome [8].

All large cytogenetic studies of AML cases showed that patients harboring t(15;17)(q22;q12-21) have an excellent prognosis and those with t(8;21)(q22;q22) or inv(16)(p13q22) have a relatively favorable prognosis. Patients exhibiting monosomies of chromosomes 5 and 7 or deletions in the long arms of these and patients with a complex karyotype, i.e. controversially defined as at least 3 or at least 5 chromosomal

aberrations, have a poor clinical outcome [28,29]. The MLL gene located at 11q23 occasionally undergoes partial tandem duplication which was demonstrated, too, to be an unfavorable prognostic factor [30].
About 45-60% of patients with AML showing a normal karyotype carry a mutation in the NPM1 (nucleophosmin 1) gene. This mutation is said to confer good prognosis, however, will be overridden by FLT3 mutations (internal tandem repeats) if existent alongside NPM1 mutations [31].
Nevertheless, the age of the patient remains the most important prognostic factor, as the outcome definitely deteriorates with increasing age [29].

1.4 Eosinophilia

Eosinophilic cells derive from myeloid progenitors and represent a subgroup of granulocytes. They play an important role in immune responses as they destroy parasites and they also contribute to the pathological mechanisms of allergic reactions [32]. Eosinophil development is considerably influenced by cytokines such as GM-CSF[4], and interleukins IL-3 and IL-5, with only the latter displaying specificity for eosinophils. The major source of the eosinophil specific IL-5 is a subset of T lymphocytes called type 2 helper T cells (T_H2) [33].
In healthy adults, circulating eosinophils constitute about 1% to 5% of the total white blood cell count (i.e. 50-400 μl^{-1}) [32]. If the count of eosinophils is elevated above the indicated number, a condition called eosinophilia arises [24].

1.4.1 Classification of eosinophilia and its causes

Depending on the degree of eosinophilic increase one distinguishes between mild (<1500 μl^{-1}), moderate (1500-5000 μl^{-1}) and severe (>5000 μl^{-1}) subtypes [34]. Furthermore, depending on its causes, eosinophilia is divided into three categories [35]:

1. reactive (non-clonal) eosinophilia
2. clonal disorder originating in the bone marrow associated with eosinophilia
3. hypereosinophilic syndrome (HES)

[4] Granulocyte-macrophage colony stimulating factor

Reactive eosinophilia results from cytokines that stimulate eosinophilic proliferation. Such cytokines, e.g. IL-5, are produced mainly by T_H2 cells, yet also eosinophils themselves secrete these molecules allowing autocrine fashion of their proliferation. Eosinophilia due to cytokines produced by T cells can be associated with all kinds of illnesses like for instance infections, allergies, skin or connective tissue diseases, cardiac or pulmonary diseases or malignancies [34,35].
In *clonal eosinophilia* a specific genetic abnormality leads to hematopoietic progenitor cell disorders in the bone marrow, e.g. chronic eosinophilic leukemia (CEL) with FIP1L1-PDGFRα fusion gene [36].
The *hypereosinophilic syndrome* (HES) is diagnosed when (a) elevated eosinophil levels (>1500 μl^{-1}) are persistent for at least six months, (b) evidence for end organ damage due to hypereosinophilia is provided, and (c) other causes of eosinophilia such as infectious, allergic or malignant disorders can be excluded. Some cases of HES have also been shown to involve the FIP1L1-PDGFRα fusion gene [33].

1.4.2 FIP1L1-PDGFRα fusion in eosinophilia

The fusion of the FIP1L1 gene to the PDGFRα gene is quite unusual as it does not happen by a chromosomal translocation but rather via a cryptic deletion of ~ 800 kb [36]. This deletion on chromosome 4q12 is not visible by routine karyotyping but can be detected by FISH techniques or reverse transcriptase PCR (RT-PCR) analysis [33,36].
The fusion involves highly variable breakpoints in FIP1L1 (scattered over introns 7-10) and less variable breakpoints in PDGFRα (restricted to exon 12) and produces the constitutively active tyrosine kinase FIP1L1-PDGFRα. The molecular mechanisms of the chimeric kinase leading to eosinophilic differentiation are not understood yet [35].
Interestingly, imatinib, approved treatment for BCR-ABL positive CML patients, also shows great success in medical therapy of FIP1L1-PDGFRα positive eosinophilias [36].

1.4.3 Clinical manifestations

Complications due to pathologically acting eosinophils involve the release of granule molecules such as eosinophil cationic protein (ECP), major basic protein (MBP), eosinophil derived neurotoxin (EDN), or enzymes like eosinophil peroxidase (EPO), elastase and collagenase [33]. Together with infiltration of tissues these granule contents provoke thrombosis, endothelial damage or neurotoxic implications. The organs most commonly damaged by eosinophil proteins are skin (predominant

complication), heart (congestive heart failure), nervous system (from memory loss to stroke) and lungs [33,34].

2 Aim of the project

2.1 Routine diagnostics

In routine laboratory diagnostics hematological disorders are adjudged due to specifically developed molecular biological and cytogenetic tests.
Material of patients with clinical suspicion of distinct hematological diseases is sent from various Austrian hospitals, from which nucleic acids of peripheral blood and bone marrow cells are prepared for molecular biological analysis. For cytogenetic studies mainly bone marrow cultures are established from which a karyogram is made for each patient which gives first hints for whether or not some chromosomal aberrations are existent. In combination with the results of the requested molecular biological tests suspicion of disease can be either confirmed or eliminated. It can also lead to further investigation, especially on the level of cytogenetics, in particular FISH studies.
Going through the whole process of analysis one is often confronted with unusual genotypes in hematological diseases. These are for example cases of leukemia with atypical translocations that have not been described yet, or translocations probably linked to some interesting forms of pathological phenotypes, i.e. strong increase in the number of eosinophilic cells.
Such diagnostic findings are not further assayed in routine work, instead these cases are subject to further investigation in separate projects.

2.2 Where routine ends and special tasks begin

The elucidation of such unusual cases was the aim of this project.
Generally, the identification of fusion genes and their resulting chimeric transcripts and proteins is of great importance in cancer, especially leukemia, therapy. Effective medical

therapeutic drugs can only be developed if the underlying mechanisms of disease origin are known. The elucidation of diverse genetic rearrangements is the first step in this very time-consuming and laborious process. If a patient's genetic make up is known, physicians can more easily apply targeted therapy.
Therefore, the identification of novel gene fusions is a very valuable aspect in clinical matter. Genetic analysis of the tumor represents the basis for future possibilities in patient specific therapy with minimal side effects.

In the first part of this project, the case of an AML patient attracted a great deal of attention when an unusual constellation of chromosomal aberrations was detected. As a consequence of translocations, distinct fusion genes are very likely to arise. Considering the existent translocation on chromosome 12p13 and comparing this case with publications concerning fusion genes in leukemia, ETV6 on chromosome 12 emerged as possible candidate gene in this rearrangement. Indeed, the transcription factor ETV6 was shown to constitute one part of the resulting fusion gene. The partner gene was to be identified and characterized with the help of both elegant and standard methods.

Secondly, the work with BACs had to be established in the laboratory.
After successful introduction of BAC methods, these, too, should be used to identify putative fusion genes that resulted from a translocation. For these cases, both of which were associated with eosinophilia, data from conventional cytogenetics, i.e. karyograms, were available only. Thus, the area of the breakpoint had to be narrowed down. BAC-FISH was the tool of choice to walk along chromosomes to define the breakpoint and identify genes involved in the rearrangement.

3 Materials and Methods

3.1 Patient histories and profiles

Patient A – male, born in 1951

In 2005 patient A was diagnosed with undifferentiated AML (M0 according to the FAB classification) due to diverse molecular biological tests, cytogenetic studies, FACS analysis, and histological examination.

The translocation t(8;21) giving rise to the AML1-ETO fusion gene often seen in AML was not detected, neither was the inversion of chromosome 16 which results in another fusion gene, CBFβ-MYH11, typical for AML.

Karyograms of unstimulated bone marrow cultures and FISH studies showed the existence of a mosaic. Two pathological cell lines were discovered, one of them exhibiting monosomy 7 and the translocation t(10;12) and the second one exhibiting monosomy 7, t(10;12) and an additional translocation between chromosome 12 and an acrocentric chromosome. The involved band on chromosome 12 was assumed as 12p13. In a previous project the transcription factor ETV6 on chromosome 12p13 was shown to constitute the 5´ part of the fusion gene.

Despite chemotherapy the patient died due to progression of disease 9 months after diagnosis.

Patient B – male, born in 1931

Due to cytological examinations in early 2008 patient B was found to show considerable increase in eosinophilic cells which constituted 60% of total bone marrow cell count. Thus, indication of hypereosinophilic syndrome arose. Additionally, a relative increase in so-called T-NK cells was present in both peripheral blood and bone marrow samples.

The translocation t(8;21)(q22;q22), one of the most frequent chromosomal abnormalities associated with AML [5], was not existent according to both molecular

biological tests and FISH studies. Moreover, the fusion gene FIP1L1-PDGFRA was detected neither in peripheral blood nor in bone marrow samples. Additionally, abnormal T cell proliferation could be ruled out due to negative results for TCR γ rearrangement PCR.
As far as cytogenetic studies are concerned, a chromosomal mosaic was found. Next to a cell line with loss of chromosome Y, which is probably attributed to advanced age, another cell line with a translocation between chromosomes 9 and 21 in addition to loss of chromosome Y was detected. The translocation was subsequently shown to be non-constitutional. Hence it was thought to be associated with the strongly increased number of eosinophils.

Patient C – male, born in 1925

In early 2008 immunological phenotyping of patient C bone marrow detected a prominent increase in eosinophilic cells to 50%. Additionally, a pathological B cell population was detected. Due to the existing eosinophilia, suspicion of hypereosinophilic syndrome, Churg Strauss syndrome[5] or eosinophilic leukemia emerged.
The fusion gene FIP1L1-PDGFRA could not be detected in peripheral blood or in bone marrow samples. The existence of a clonal B cell population was proven by PCR analysis of Ig heavy chain rearrangements.
Cytogenetic studies revealed a translocation t(4;13)(q35;q12) as mosaic with loss of chromosome Y, the latter event being probably attributed to advanced age. The translocation was thought to be associated with the increase in eosinophilic cells and was therefore subject to further investigation to identify genes possibly playing a role in the pathological process.

3.1.1 Patient material

For the diploma thesis at hand previously prepared material of the above patients was used. On the one hand extracted nucleic acids and on the other hand bone marrow cells in Carnoy's fixative (3:1 methanol to glacial acetic acid) were available.

[5] A type of vasculitis characterized by pulmonary infiltrates.

3.2 Molecular Biological Methods

Unless otherwise indicated, Aqua Bidest. "Fresenius" (Fresenius Kabi) or alternatively Ecotainer® Aqua B. Braun (B. Braun) was used for all reactions and is designated as ddH_2O only.

3.2.1 Bacterial strain

For cloning experiments the chemically competent TOP10 *E.coli* strain (Invitrogen) was used.

3.2.2 Media and culture plates

For production of selective liquid media and selective culture plates kanamycin sulfate (Kan; SIGMA®) or chloramphenicol (CAM; SIGMA®) was added in a working concentration of 50 µg/ml or 12,5 µg/ml, resp. Both stock solutions, the 10 mg/ml kanamycin (in ddH_2O) and the 25 mg/ml chloramphenicol (in EtOH absolute, Merck), were sterile filtered using a 0,2 µm filter (Sarstedt).

LB liquid medium	10 g LB Broth Base (SIGMA®) 500 ml ddH_2O autoclave for 15 minutes at 121°C store at 4°C
LB agar	16 g LB Agar (SIGMA®) 500 ml ddH_2O autoclave for 15 minutes at 121°C store at 4°C

3.2.3 cDNA synthesis

For cDNA synthesis 2 µg RNA were diluted in DEPC treated ddH_2O to a final volume of 11 µl. After adding 1 µl 250 ng/µl Random Primer (Amersham Biosciences) the reaction was incubated at 65°C for 5 minutes, chilled on ice for another 5 minutes and briefly centrifuged. Now 1 µl 10 mM dNTP mix (Amersham Biosciences), 6 µl M-MLV RT 5x Buffer (Promega), 0,5 µl Rnasin® Plus (40 U/µl; Promega) and 1 µl M-MLV RT, Rnase

(H-) Point Mutant (200 U/µl; Promega) were added, mixed and incubated at 42°C for one hour. The reaction was stopped by incubation at 70°C for 15 minutes, then it was cooled on ice, briefly centrifuged, mixed and could be stored at -20°C until further use.

DEPC treated ddH_2O	0,1% DEPC (SIGMA®) in ddH_2O shake well and incubate over night at 37°C autoclave and store at room temperature

3.2.4 Polymerase chain reaction (PCR)

The following PCRs were run either in the Mastercycler ep gradientS (Eppendorf) or alternatively in the PCR Express thermal cycler (Hybaid). The primers used for PCR (and sequencing, see 3.2.13) were designed with the help of Gene Runner Version 3.05 (Copyright © 1994 Hastings Software, Inc.) and the online available software Primer3 [37] and the Oligo Java-Applet on the TIB-MolBiol Homepage (OLIGO-Applet © 1998 by O.Landt/M.Peters-TIB Molbiol Berlin). The primers were ordered at and synthesized by Applied Biosystems, Metabion, TIB Molbiol or genXpress. For primer sequences refer to Appendix.

3.2.4.1 Amplification of unknown cDNA ends

In order to amplify cDNA strands with unknown 3´ ends a technique called 3´RACE PCR was performed using the GeneRacer™ Kit (Invitrogen, Austria).

The mRNA's PolyA tail is exploited when Oligo dT primers are annealed to the cDNA and reverse transcription is performed. As the Oligo dT primer has a defined 36 nucleotide sequence attached to it, regular PCR can be performed with the gene specific forward primer and a reverse primer complementary to the attached sequence of the Oligo dT primer. For increased specificity nested PCR was run subsequently.

For cDNA synthesis 1 µg of total RNA was used. 1 µl GeneRacer™ Oligo dT Primer (50 µM), 1 µl dNTP mix (10 mM each), and DEPC treated water ad 13 µl were added.
The mixture was incubated at 65°C for 5 minutes, chilled on ice for > 1 minute and centrifuged briefly.
Four µl 5x First Strand Buffer, 1 µl 0,1 M DTT, 1 µl RnaseOut (40 U/µl) and 1 µl SuperScript III RT (200 U/µl) were added to reach a final volume of 20 µl. After

incubation at 50°C for 45 minutes, the reaction was stopped by incubation at 70°C for 15 minutes. The mix was chilled on ice, centrifuged briefly at maximum speed and 1 µl RnaseH (2 U) was added. RNA digestion occurred for 20 minutes at 37°C. Now cDNA was ready for RACE PCR.

Three separate reactions were run in order to cover up a wider part of the ETV6 transcript sequence. Therefore, RACE PCR was performed using the GeneRacer primer and one of three different gene specific primers (ETV6A, ETV6B, ETV6C) each. The primers were designed with regard to the major isoform of ETV6 (for transcript sequence information refer to Appendix).
For the reaction 1 µl cDNA, 3 µl 10 µM GeneRacer™ 3´ primer, 1 µl 10 µM ETV6 primer, 5 µl 10x High Fidelity PCR Buffer (Invitrogen), 1 µl 10 mM dNTP mix, 0,5 µl Platinum® *Taq* DNA Polymerase High Fidelity (5 U/µl; Invitrogen), 2 µl 50 mM $MgSO_4$ and ddH_2O were added to a final volume of 50 µl.

Table 3.1 Hot start and touchdown PCR program used for RACE PCR

Step	***Time, Temperature***	***Cycles***
Initial denaturation	2 min at 94°C	1
Denaturation Annealing, extension	30 sec at 94°C 2 min at 72°C	5
Denaturation Annealing, extension	30 sec at 94°C 2 min at 70°C	5
Denaturation Annealing Extension	30 sec at 94°C 30 sec at 65°C 2 min at 68°C	25
Final extension	10 min at 68°C	1

For nested PCR 1 µl of 1:100 diluted initial PCR product, 1 µl 10 µM GeneRacer™ 3´ nested primer, 1 µl 10 µM ETV6 nested primer (ETV6Anested, EVT6Bnested or ETV6Cnested), 5 µl 10x High Fidelity PCR Buffer (Invitrogen), 1 µl 10 mM dNTP mix, 0,5 µl Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen; 5 U/µl), 2 µl 50 mM $MgSO_4$ and ddH_2O were mixed in a final volume of 50 µl.

Table 3.2 PCR program used for nested RACE PCR

Step	***Time, Temperature***	***Cycles***
Initial denaturation	2 min at 94°C	1
Denaturation Annealing Extension	30 sec at 94°C 30 sec at 65°C 2 min at 68°C	25
Final extension	10 min at 68°C	1

3.2.4.2 PCR on a plasmid DNA template

The RACE PCR products were cloned into the pCR®4-TOPO® vector (Invitrogen) from which the fragments between the M13 Reverse and M13 Forward (-20) priming sites could be amplified for subsequent sequencing.

0,5 – 1 ng plasmid DNA, 5 µl 10x PCR Gold Buffer (Applied Biosystems), 5 µl 25 mM $MgCl_2$ (Applied Biosystems), 1 µl 10 mM dNTP mix (Amersham Biosciences), 1 µl 10 pmol/µl M13F primer (Applied Biosystems), 1 µl 10 pmol/µl M13R primer (Applied Biosystems), 0,4 µl AmpliTaq Gold™ DNA Polymerase (5 U/µl; Applied Biosystems) and ddH_2O were mixed in a final volume of 50 µl.

Table 3.3 PCR program used for amplification from a plasmid template

Step	***Time, Temperature***	***Cycles***
Initial denaturation	2 min at 94°C	1
Denaturation Annealing Extension	1 min at 94°C 30 sec at 55°C 2 min at 72°C	25
Final extension	10 min at 72°C	1
Pause	∞ at 12°C	-

3.2.4.3 Reverse transcriptase PCR (RT-PCR)

For RT-PCR experiments cDNA was used non-diluted and was mixed with 5 µl 10x PCR Gold Buffer (Applied Biosystems), 5 µl 25 mM $MgCl_2$ (Applied Biosystems), 1 µl 10 mM dNTP mix (Amersham Biosciences), 1 µl 10 pmol/µl forward primer, 1 µl 10 pmol/µl reverse primer, 0,4 µl AmpliTaq Gold™ DNA Polymerase (5 U/µl; Applied Biosystems) and ddH_2O to reach a final volume of 50 µl.

Table 3.4 PCR program used for reverse transcriptase PCR experiments

Step	***Time, Temperature***	***Cycles***
Initial denaturation	2 min at 94°C	1
Denaturation Annealing Extension	30 sec at 94°C 30 sec at 55°C 1 min at 72°C	35
Final extension	10 min at 72°C	1
Pause	∞ at 12°C	-

All PCR products were analyzed by agarose gel electrophoresis.

3.2.5 Agarose gel electrophoresis

Agarose gel electrophoresis served a double purpose. Firstly, to separate fragments according to size, and secondly, to estimate the DNA amount in the PCR reaction for subsequent sequencing analysis.
Depending on the size of the PCR products 1% or 2% agarose gels were made. Agarose MP (Roche) was dissolved in 0,5x TBE (SIGMA®) by heating in the microwave. After cooling, ethidium bromide (10 mg/ml, Amresco) was added in a working concentration of 0,5 µg/ml.

The samples were mixed with 6x Loading Dye and loaded on the gel. Depending on the expected band sizes, the O´RangeRuler™ 200 bp DNA Ladder (Fermentas), pUC19 Hpa II DNA Marker (Fermentas) or the MassRuler™ DNA Ladder, Low Range (Fermentas) was used as marker and the gel was run in 0,5x TBE at 110 – 140 V depending on the size of the gel chamber. For illustration of the markers refer to Appendix.

3.2.6 Gel extraction

The bands of interest of the nested RACE PCR were excised from the agarose gel with a fresh razor blade for each band. The gel piece was transferred onto the S.N.A.P. columns included in the GeneRacer™ Kit. Depending on the size of the gel piece, 20 - 60 µl DNA were collected in a microcentrifuge tube by centrifugation for 1 minute at 13.000 rpm (Heraeus Biofuge Pico) and could now be stored at -20°C until further use.

3.2.7 TOPO TA cloning

Gel extracted nested RACE PCR products were cloned into the pCR®4-TOPO® vector using the TOPO TA Cloning® Kit for Sequencing (Invitrogen).

The pCR®4-TOPO® vector used for cloning is linearized with single 3´ thymidine (T) overhangs and a topoisomerase covalently bound to it (also referred to as "activated" vector). *Taq* polymerase used in previous PCR amplifications has a nontemplate-dependent terminal transferase activity which adds a single deoxyadenosine (A) to the 3´ ends of PCR products. This allows PCR products to ligate with the vector via A-T base pairing.

Viral topoisomerase I binds specifically to duplex DNA and cleaves one strand after 5´-CCCTT. The energy from the broken phosphodiester backbone is conserved by a covalent bond between the 3´ phosphate of the cleaved DNA strand and a tyrosyl residue (Tyr-274) of the enzyme. The phospho-tyrosyl bond can subsequently be attacked by the 5´ hydroxyl group of the original cleaved strand which reverses the reaction and releases the topoisomerase enzyme. TOPO® Cloning exploits this reaction to efficiently ligate PCR products with the vector [38], see figure 3.1.

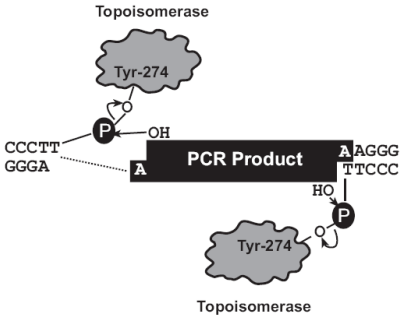

Figure 3.1 *Activated vector in ligation process. Interaction between vector DNA, PCR product and Topoisomerase I during cloning process is demonstrated (figure from [38]).*

The use of the pCR®4-TOPO® vector allows direct selection of recombinants as it contains the lethal *E.coli* gene *ccdB* fused to the C-terminus of the *LacZ*α fragment. If a PCR product is successfully cloned into the vector the expression of the *LacZ*α*–ccdB* gene fusion is disrupted thus permitting growth of only recombinants upon transformation of TOP10 cells [38]. For pCR®4-TOPO® vector map refer to Appendix.

The cloning reaction consisting of 4 µl PCR product, 1 µl Salt Solution and 1 µl TOPO® vector was mixed gently and incubated at room temperature for 30 minutes. The reaction was kept on ice until proceeding with the transformation.

3.2.8 Transformation of One Shot® TOP10 chemically competent *E.coli*

Transformation of competent cells was performed with reagents included in the TOPO TA Cloning® Kit for Sequencing (Invitrogen).

After thawing the competent cells (50 µl) on ice, 2 µl of the cloning reaction were added, gently mixed and incubated on ice for 5-30 minutes. After a heat shock at 42°C

for 30 seconds the cells were immediately put on ice. The transformed cells were mixed with 250 µl room temperature S.O.C. medium and shaken for one hour at 37°C.
The cells were cultivated on pre-warmed LB/Kan plates over night at 37°C in an incubator with natural convection, type BD53 (Binder).

3.2.9 Plasmid DNA isolation

All incubation steps of cells in liquid medium were done in a water bath type 1092 (GFL) whereas culture plates were incubated in an incubator with natural convection, type BD53 (Binder).

3.2.9.1 Plasmid mini preparation using the TENS method

All centrifugation steps in this procedure were done at 4°C in the Centrifuge 5415R equipped with the rotor F-45-24-11 (Eppendorf).
A single colony was picked with a sterile toothpick and 5 ml LB/Kan medium were inoculated. The cells were grown over night at 37°C with vigorous shaking (~ 220 rpm).
Cells were harvested by centrifugation of 1,5 ml of the overnight culture at 13.200 rpm for 30 seconds. The supernatant was discarded and the pellet was resuspended in the residual medium. Three-hundred µl TENS were added and mixed thoroughly. In the next step 150 µl 3 M NaOAc pH 5,2 were added, mixed and centrifuged for 5 minutes at 13.200 rpm. The supernatant was now transferred to a new tube and 900 µl ice cold EtOH absolute (Merck) were added and mixed. The DNA was pelleted by centrifugation at maximum speed for 30 minutes and washed twice with 1 ml 70% EtOH each.
EtOH was discarded and the pellet was dried at ambient temperature under vacuum in the UNIVAPO 100 H (UniEquip) for about 20 minutes. Finally, the dry pellet was dissolved in 100 µl TE buffer.

3 M NaOAc	20,4 g NaOAc · 3 H_2O (Merck)
	add 20 ml ddH_2O
	adjust pH to 5,2 using glacial acetic acid (Merck)
	ad 50 ml with ddH_2O
TENS	0,1 N NaOH (Merck)
	0,5 % SDS (SIGMA®)
	ad 50 ml with TE pH 8,0 (MP Biomedicals)

3.2.9.2 BAC DNA isolation

Generally, BACs are plasmids that contain a chromosomal DNA insert of up to 150 kb or even more. For our purposes BACs containing specific human genomic sequences were used.
BACs were ordered at the BACPAC Resources Center at Children's Hospital Oakland Research Institute (CHORI) in California, USA. They arrived as LB stabs containing 12,5 µg/ml chloramphenicol. The DH10 *E.coli* host harbors the genomic fragment of interest cloned into either the pBACe3.6 or pTARBAC2 vector. For vector maps and a complete table of BACs used refer to Appendix.

BAC DNA was isolated using the QIAGEN® Plasmid Midi Kit (QIAGEN) in combination with the User-developed Protocol.

All centrifugation steps in this procedure were performed at 4°C in the centrifuge ROTANTA/TR (Hettich).

A sterile toothpick was used to inoculate 5 ml LB/CAM medium with cells from the LB stab. The cells were grown over night at 37°C and 220 rpm. The next day the over night culture was streaked out on LB/CAM plates and incubated over night at 37°C.
The next morning a single colony was picked and 5 ml LB/CAM were inoculated to grow a starter culture at 37°C and 220 rpm. Five-hundred µl of the starter culture were used in the evening to grow an over night culture in 100 LB/CAM at 37°C and 230 rpm.
The next day, the cells were divided into two 50 ml tubes and harvested by centrifugation at 4500 rpm for 20 minutes.
Each cell pellet was resuspended completely in 10 ml Buffer P1 (containing RnaseA in a concentration of 100µg/ml) by pipetting up and down and by harsh vortexing.
For cell lysis 10 ml Buffer P2 were added to each tube, mixed thoroughly but gently by inverting 4-6 times and incubated at room temperature 5 minutes.
Then 10 ml chilled Buffer P3 were added to each tube, immediately mixed by gently inverting 4-6 times, and incubated on ice for 15 minutes.
Centrifugation at 4500 rpm for 30 minutes pelleted the cell debris and the supernatant containing the plasmid was removed as quickly as possible. The supernatant was centrifuged again at 4500 rpm for 15 minutes and the supernatants of both tubes were removed and pooled. Meanwhile, one QIAGEN-tip 100 for each over night culture was equilibrated by applying 4 ml Buffer QBT and the column was allowed to empty by gravity flow.

After equilibration the supernatant was applied to the QIAGEN-tip 100 and was allowed to enter the resin by gravity flow.
Subsequently, the column was washed twice with 10 ml Buffer QC each and DNA was eluted with 5x 1 ml of prewarmed (65°C) Buffer QF.
DNA was precipitated by adding 3,5 ml room temperature isopropanol, mixing by inverting and centrifugation at 4500 rpm for 1 hour. The pellet was washed with 2 ml of room temperature 70% EtOH and centrifuged at 4500 rpm for 30 minutes.
Finally, the pellet and was air-dried for 5-10 minutes and dissolved in a suitable volume (30 – 70 µl) of TE buffer.

Plasmid DNA concentration of both mini preparations and BAC DNA isolations was measured in an Ultraspec 2000 photometer (Pharmacia Biotech) and calculated according to the following formula:

$$\frac{OD_{260} * 50 * dilution\ factor}{1000} = concentration\ [\mu g/\mu l]$$

Before continuation of work with extracted BAC DNA verification by sequencing was suggested by BACPAC Resources to make sure the clone stock they send is pure as their libraries have little to well contamination.

3.2.10 Bacterial glycerol stocks

All steps were done under sterile conditions.
One ml of an over night culture and 500 µl autoclaved 87% glycerol (Merck) were mixed in a cryo tube by vortexing until a homogenous mixture was formed. The glycerol stock was stored at -70°C (GFL freezer).

3.2.11 Mutational analysis

For a complete profile of patient A's medical history, he was belatedly tested for two more typical AML mutations involving the NPM1 and FLT3 genes. From a clinically point of view these examinations did not fulfill any purpose since the patient had already deceased.

Mutations in exon 12 of the NPM1 gene result in an alteration of the amino acid tryptophan at position 288. This causes a change in the nuclear localization signal with consequent cytoplasmic accumulation of the NPM1 phosphoprotein.
The favorable clinical outcome of NPM1 mutations in patients with normal karyotype will be overridden by concomitantly existing FLT3 ITD (internal tandem duplication) mutations [31].
FLT3 is a tyrosine kinase that shows constitutive activation status in patients with the ITD mutation. These patients carry a sequence alteration in the juxtamembrane (JM) portion of the protein. As a result, increased proliferation and reduced susceptibility to apoptosis in hematopoietic cells arise [39]. FLT3 kinase inhibitors are available as therapy and are therefore an option for medical treatment.

The following procedures were performed according to protocols internally developed for routine diagnostics. PCR amplifications were run in the Mastercycler ep gradientS (Eppendorf).

3.2.11.1 NPM1 mutation detection

For reverse transcriptase PCR, 2 µl of both available cDNAs were mixed with 15,3 µl ddH_2O, 2,5 µl 10x PCR Gold Buffer (Applied Biosystems), 2,5 µl 25 mM $MgCl_2$ (Applied Biosystems), 0,5 µl 10 mM dNTP mix (Amersham Biosciences), 1 µl 10 pmol/µl NPM1b-for primer, 1 µl 10 pmol/µl NPM1b-rev primer, and 0,2 µl AmpliTaq Gold™ DNA Polymerase (5 U/µl; Applied Biosystems) in a final volume of 25 µl.

Table 3.5 PCR program used for NPM1 mutation detection

Step	***Time, Temperature***	***Cycles***
Initial denaturation	5 min at 94°C	1
Denaturation Annealing Extension	30 sec at 94°C 30 sec at 60°C 30 sec at 72°C	40
Final extension	4 min at 72°C	1
Pause	∞ at 12°C	-

The PCR product was subject to sequencing analysis.

3.2.11.2 FLT3-ITD detection

For PCR amplification 2 µl DNA and 0,4 µl AmpliTaq Gold™ DNA Polymerase (5 U/µl; Applied Biosystems) were mixed with 50 µl of a previously prepared mix containing 37,9 µl ddH_2O, 5,3 µl 10x PCR Gold Buffer (Applied Biosystems), 5,3 µl 25 mM $MgCl_2$ (Applied Biosystems), 1,1 µl 10 mM dNTP mix (Amersham Biosciences), 0,2 µl 100 pmol/µl ITD F primer, and 0,2 µl 100 pmol/µl ITD R primer.

Table 3.6 PCR program used for FLT3-ITD detection

Step	***Time, Temperature***	***Cycles***
Initial denaturation	10 min at 95°C	1
Denaturation Annealing Extension	30 sec at 95°C 30 sec at 54°C 30 sec at 72°C	40
Final extension	min at 72°C	1
Pause	∞ at 12°C	-

For detection of a possibly existent mutation the PCR product was analyzed with the help of DHPLC (denaturing high pressure liquid chromatography, Transgenomic®). The 'sizing program' of the associated WAVE® System 4500 was used which does not depend on denaturing conditions. Dependent on whether the internal tandem repeats are existent the chromatogram will show one peak for wildtype DNA or several peaks for mutated DNA. As ITDs increase the length of the target region products of different sizes emerge in the PCR amplification and are detected in the DHPLC UV detector sequentially according to their size differences, underlying the same principle as in gel electrophoresis. Results are converted to digital data by the Navigator™ Software.

3.2.12 Sequencing analysis

3.2.12.1 Purification of PCR products

PCR products subject to sequencing analysis had to be purified first by using the ExoSAP-IT® system (USB).

The ExoSAP-IT reagent is used for clean-up of PCR reactions that are used in downstream applications such as DNA sequencing. After completion of PCR unconsumed dNTPs and primers must be removed from the PCR product mixture as they would interfere with the sequencing method. ExoSAP-IT removes these contaminants by the use of two hydrolytic enzymes: Exonuclease I and shrimp alkaline phosphatase.

Exonuclease I removes any single-stranded DNA including unused primers while shrimp alkaline phosphatase converts the remaining dNTPs into inorganic phosphates [40].
One reaction consisted of 5 µl PCR product and 2 µl ExoSAP-IT and was run in the Mastercycler ep gradientS (Eppendorf).

Table 3.7 Program used for ExoSAP-IT purification

Step	Time, Temperature
Purification reaction	15 min at 37°C
Inactivation of enzyme	15 min at 80°C
Pause	∞ at 12°C

If a greater volume of product is needed the reaction can also be carried out with 10 µl PCR product and 4 µl ExoSAP-IT reagent.

3.2.12.2 Sequencing reaction

The DNA template was subsequently processed in the sequencing reaction using the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems).

Cycle sequencing is very similar to PCR since successive rounds of denaturation, annealing and extension give rise to amplified extension products. Thereby fluorescent fragments are produced by incorporation of labeled ddNTPs. Each different ddNTP (ddATP, ddCTP, ddGTP, or ddTTP) carries a different fluorescent dye and so all terminated fragments exhibit a dye at their 3´ end [41].

3.2.12.2.1 Sequencing of ExoSAP purified PCR products

For each sample two reactions were performed since sequencing was done separately for both forward and reverse primers. The same primers were used as in the previous PCR amplification. For primer sequences refer to Appendix.

The required volume of the ExoSAP-IT purified PCR product for the sequencing reaction depends on the size and the concentration of the amplified fragment. The required amount (in ng) is calculated according to a formula that was laboratory internally developed:

$$\frac{\textit{fragment length (in bp)}}{100} = \textit{required amount (in ng)}$$

The amount of DNA in the PCR product mixture was estimated with the help of the gel picture or with the help of DHPLC (Transgenomic®). Subsequently the concentration [ng/µl] could be determined and finally the required volume of PCR product is calculated:

$$\frac{\textit{required amount (in ng)}}{\textit{estimated concentration [ng/µl]}} = \textit{required volume (in µl)}$$

3.2.12.2.2 Direct sequencing of BAC DNA

For verification of BAC clones extracted DNA was used directly in sequencing reactions as no prior PCR and ExoSAP-IT purification were performed.

Applied Biosystems recommend 1 µg of BAC DNA for sequencing analysis. As the genomic DNA in the vector is flanked by the SP6 and T7 sites direct sequencing with primers complementary to these sites is possible. For vector maps and primer sequences refer to Appendix.

For the sequencing reaction 14-x µl ddH_2O, x µl DNA, 2 µl BigDye® Terminator v1.1 Ready Reaction Mix, 3 µl BigDye® Terminator v1.1 5x Sequencing Buffer and 1 µl 5 pmol/µl primer (Applied Biosystems) were mixed. The reaction was run in the Mastercycler ep gradientS (Eppendorf).

Table 3.8 Program used for all sequencing reactions

Step	***Time, Temperature***	***Cycles***
Initial denaturation	30 sec at 96°C	1
Denaturation Annealing Extension	10 sec at 95°C 5 sec at 50°C 3 min at 60°C	25
Pause	∞ at 12°C	-

3.2.12.3 Purification of the sequencing reaction

The samples had to undergo a final purification step before they could be loaded on the genetic analyzer.

Unincorporated fluorescently labeled ddNTPs and salt would interfere with the sequencing analysis and produce poor signal-to-noise ratios. The BigDye® Xterminator™ Purification Kit (Applied Biosystems) is used for clean-up which captures the contaminants by means of specially developed beads [42].

For purification 45 µl SAM Solution and 10 µl Xterminator Solution were mixed and added to 10 µl of the sequencing reaction in a MicroAmp Optical 96-well Reaction Plate (Applied Biosystems). The plate is reversibly sealed and vortexed for 30 minutes at 2000 rpm using the MixMate PCR 96 (Eppendorf).
Prior to loading the plate on the Genetic Analyzer it needed to be centrifuged for 2 minutes at 3000 rpm in the Labofuge 400e (Heraeus) so that the beads are collected at the bottom of the well and the capillaries of the sequencer would not encounter the Xterminator Solution containing the contaminants.

3.2.12.4 ABI 3130 Genetic Analyzer

After completion of the final purification step the samples were ready for analysis based on capillary electrophoresis in the ABI 3130 Genetic Analyzer (Applied Biosystems).

During capillary electrophoresis the products of the sequencing reaction are injected into capillaries that are filled with polymer (POP-6™ polymer, Applied Biosystems). An electrical field is established which makes the negatively charged DNA fragments move through the polymer towards the positive electrode according to their size.
Before reaching the positive electrode the DNA fragments pass a laser beam which causes them to fluoresce. The fluorescence signal is detected by an appropriate detector and is converted to digital data by the 3130 Data Collection Software (see figure 3.2) [43].

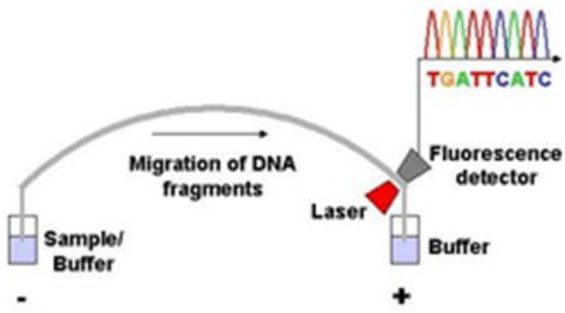

Figure 3.2 *Working principle of the genetic analyzer. The migration of DNA fragments in the electrical field toward the laser beam and fluorescence detector are illustrated (picture from [43]).*

Prior to starting the genetic analyzer the associated software was used to enter the samples' specifications and to adapt the appropriate settings.
The MicroAmp Optical 96-well Reaction Plate was placed in the designated tray and the specific program could be started.

Data analysis was done with the Applied Biosystems Sequencing Analysis v5.2 Patch2, or SeqScape® v2.5 software. The obtained sequence was used for BLAST (Basic Local Alignment Search Tool, NCBI) search on the freely accessible website http://www.ncbi.nlm.nih.gov/blast/Blast.cgi.

3.2.13 Production of Fluorescently Labeled Probes

BAC DNA that was previously isolated had to be fluorescently labeled so that it could be used as probe in FISH experiments. Labeling was carried out with the Nick Translation Kit (Abbott Molecular Inc.).
This kit incorporates SpectrumGreen™- and SpectrumOrange™-directly labeled dUTPs into strands of DNA.

3.2.13.1 Nick translation

For the labeling reaction nuclease-free water, 1 µg (≤ 17,5µl) extracted BAC DNA, 2,5 µl 0,2 mM fluorescently labeled dUTP, 5 µl 0,1 mM dTTP, 10 µl 0,1 mM dNTP mix, 5 µl 10x nick translation buffer and 10 µl nick translation enzyme were mixed in a final volume of 50 µl. The reaction was briefly centrifuged, vortexed and run in the PCR Express thermal cycler (Hybaid).

Table 3.9 Program used for the labeling reaction

Step	Time, Temperature
Labeling reaction	16 hours at 15°C
Reaction stop	10 min at 70°C
Cooling	∞ at 4°C

3.2.13.2 Precipitating the probe

For precipitation of the probe 5 µl of the nick translation reaction (~ 100 ng labeled DNA), 1 µg human COT-1 DNA® (GibcoBRL), 2 µg DNA sodium salt from salmon testes (SIGMA®), 4 µl ddH_2O, 0,1 volume 3 M NaOAc, ph 5,5, and 2,5 volumes EtOH absolute (Merck) were mixed, briefly vortexed and DNA was precipitated at -70°C (GFL) for 20 minutes. After centrifugation at 13.200 rpm and 4°C for 30 minutes in the Centrifuge 5415R (Eppendorf) the pellet was dried under vacuum (UNIVAPO 100 H, UniEquip) at ambient temperature for 12 minutes. Finally, the pellet was resuspended in 3 µl ddH_2O and 7 µl Vysis® LSI/WCP® Hybridization Buffer (Abbott Molecular Inc.).
The probe could now be stored at -20°C or used right away in hybridization assays.
Before using the probe on the patient material of interest it was first tested on patient chromosomes that did not show any structural or numeral abnormalities.

3 M NaOAC, pH 5,5	20,4 g $NaOAc \cdot 3\ H_2O$ (Merck)
	add 30 ml ddH_2O
	adjust pH to 5,2 with glacial acetic acid (Merck)
	ad 50 ml with ddH_2O

3.3 Cytogenetics

For cytogenetic studies previously prepared bone marrow cells were used. For each patient cells of two differently treated bone marrow cultures were available: synchronized and non-synchronized cells.
For synchronization bone marrow cultures are treated with methotrexate (MTX). MTX is a folic acid antagonist which arrests cells during S-phase by reduction of free thymidine and thus by inhibiting DNA synthesis [44, 45]. MTX activity is neutralized by addition of leucovorin (folinic acid) and thymidine on the next day. Subsequent addition of colcemid arrests cells in metaphase by blocking microtubule spindle formation.
Non-synchronized cells are not treated with MTX but cultivated over night and finally arrested in metaphase by colcemid addition.
Both kinds of cultures are finally hypo-osmotically shocked with KCl and stored in Carnoy's fixative. Cells in fixative can be stored at -20°C for several years.

For hybridization assays, cells were dropped onto slides that have previously been washed in 70% EtOH and were stored in ddH_2O at 4°C.

3.3.1 Whole Chromosome Paints (WCP)

For whole chromosome paints XCP Human Chromosome Paints (Metasystems, Germany) were used. The probes are fluorescently labeled either with FITC or Texas Red®.

The slides were removed from the ddH_2O container and cells were dropped from about 30 cm distance when the slides were still wet. For nice spreading of inter- and metaphases the slide was breathed upon before incubation on a wet 38°C heating plate for a few minutes.
The back of the slide was dried with paper towel and the hybridization area was marked with a diamond pen.
Now 10 µl of the appropriate probe were applied. If two different probes were used 5 µl of each probe were mixed, briefly centrifuged and applied to the slide. To avoid drying-out of the hybridization area a coverslip was tightly, albeit reversibly, attached to the slide with Fixogum Rubber Cement (Marabu).
The hybridization reaction was carried out in a humidified Hybrite™ machine (Vysis) using a specific program.

Table 3.10 Hybrite program used for whole chromosome paints

Step	Time, Temperature
Denaturation	4 min at 75°C
Hybridization	< 24 hours at 37°C

The following day the coverslips were removed and the slides were washed for two minutes in wash solution 1 (73°C), for one minute in wash solution 2 (room temperature), and rinsed under ddH_2O.
After drying in the darkness 10 µl DAPI II counterstain (125 ng/ml, Abbott Molecular) or DAPI Antifade ES (125 ng/ml, Cytocell) and a coverslip were applied.
The hybridization could now be visualized using an Axioplan2 imaging fluorescence microscope (Zeiss) equipped with filter sets for FITC, Texas Red, and 4',6-Diamidino-2-phenylindol (DAPI), and images were taken with a Diagnostic Instruments D10ZNC camera using the Isis FISH imaging system software (MetaSystems).

The slides could be stored at 4°C.

Wash solution 1	0,4x SSC 0,3 % IGEPAL® CA-630 (MP Biomedicals) adjust pH to 7,0-7,5 with NaOH store at room temperature up to 6 months
Wash solution 2	2x SSC 0,1 % IGEPAL® CA-630 (MP Biomedicals) adjust pH to 7,0-7,5 with NaOH store at room temperature up to 6 months

3.3.2 Hybridization of self-labeled BACs

3.3.2.1 Hybridization according to manufacturer's instruction

Dropping of the cells was done as described for whole chromosome paints. The slides were then denatured in 75°C denaturation solution for 5 minutes.
Denaturation of precipitated BAC probes at 73°C for 5 minutes and dehydration of denatured slides for one minute each in 70%, 85% and 100% EtOH were done concomitantly.
The slides were then placed on a 38°C heating plate so that remaining ethanol would evaporate.
Finally, 10 µl probe or alternatively 5 µl of two differently labeled probes were applied to the hybridization area on the slide. To avoid drying-out of the hybridization area a coverslip was tightly, albeit reversibly, attached to the slide with Fixogum Rubber Cement (Marabu).
For hybridization the slide was placed in a humidified box in a 37°C incubator with natural convection, type BD53 (Binder) for 16 hours.

The following day the coverslips were removed, the slides were placed in 73°C wash solution 1 (see WCP), agitated for 3 seconds and incubated for 2 minutes. The second wash step was carried out in room temperature wash solution 2 (see WCP). Again, the slide was agitated for 3 seconds and incubated in the wash solution for 1 minute. Application of DAPI and visualization of the fluorochromes was performed as described for whole chromosome paints.

Denaturation solution	2x SSC 70% formamide (Merck) adjust pH to 7,0–8,0 with HCl store at 4°C up to 2 weeks

3.3.2.2 Altered hybridization procedure for more convenience

In order to possibly adopt the hybridization of self-labeled BACs for routine work a more convenient performance of this procedure was established.

Slides were prepared and dropped as described for whole chromosome paints. Easy hybridization in the Hybrite™ (Vysis) succeeded with the program for locus specific probes.

Table 3.11 Program used for hybridization of self-labeled BACs

Step	Time, Temperature
Denaturation	4 min at 75°C
Hybridization	< 24 hours at 37°C

Washing, application of DAPI and visualization of the fluorochromes were performed as described for whole chromosome paints.

4 Results

4.1 Patient A: Identification of the ETV6 partner gene

The first approach was the identification of a putative partner gene that is fused to the ETV6 locus on chromosome 12 due to the translocation found in patient A.

ETV6 was found to be one part of the fusion gene in a previous project by applying a dual color break apart probe for this gene locus. This probe consists of two differently labeled halves and is designed to prove a split in the target chromosomal area. For demonstrative reasons a picture of the LSI® ETV6 (TEL) Probe Region 12p13 (Abbott Molecular Inc.) is shown in figure 4.1.

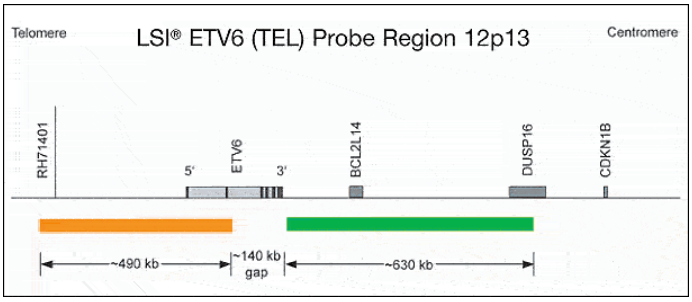

Figure 4.1 *ETV6 dual color break apart probe (picture from [46]).*

The probe was applied to fixed bone marrow cells treated with MTX and visualization proved a split between the differentially labeled halves of the probe (figure 4.2).

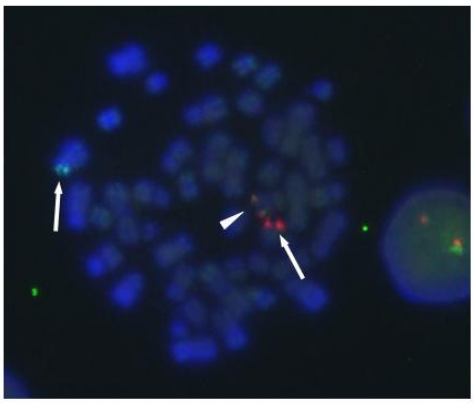

Figure 4.2 *FISH using ETV6 break apart probe. It clearly shows a split signal for one allele indicated by two arrows. The arrow head marks the fusion signal of the wildtype allele.*

In order to find the partner gene that obviously fused to ETV6 on the 3´end, RACE PCR was carried out in the next step.

4.1.1 Amplification of the unknown fusion gene with ETV6 on the 5´ end

RACE PCR is a nice method to amplify a DNA fragment of which part of the sequence is unknown. It was the method of choice in this matter and primers were designed for the already elucidated ETV6 gene.

Two independently synthesized cDNAs, yet from the same RNA template, were available so the experiment was performed with both cDNAs in case one of them is of bad quality. Next to a positive (template and primers included in the GeneRacer^{TM} kit) and a negative control (without template) three PCR amplifications for each cDNA were performed. Three distinct primers plus appropriate nested primers were designed. They were located in coding exon 2, coding exon 4, and coding exon 5 reaching into coding exon 6.
PCR amplification products analyzed by agarose gel electrophoresis are shown in figure 4.3.

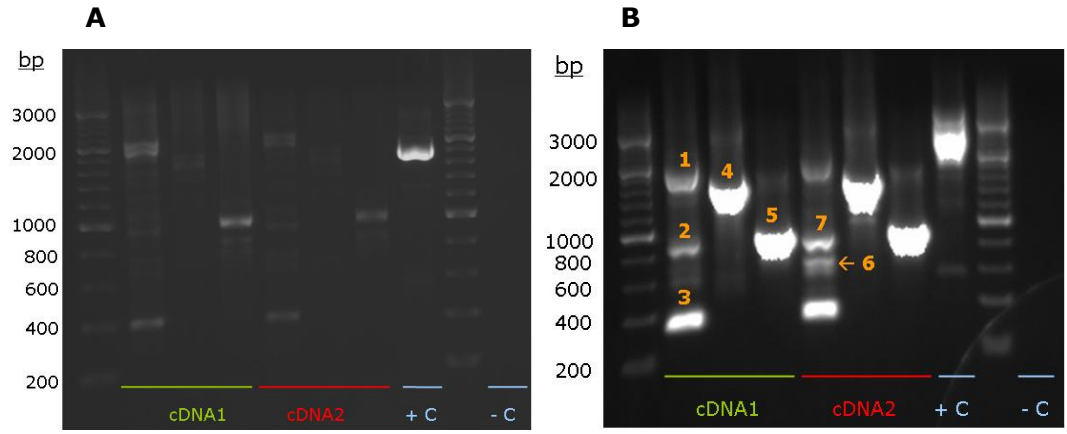

Figure 4.3 A, B *3´ RACE PCR. The result of the first RACE PCR is shown in A, whereas the nested RACE PCR is shown in B where the bands excised for further experiments are marked with numbers 1 to 7. +C, positive control; -C, negative control.*

Bands marked with numbers 1 to 7 (figure 4.3 B) were excised, DNA was extracted from the gel piece and used for subsequent cloning and sequencing.

4.1.2 Searching for clones with the fragment of interest

After plating of the *E.coli* cells transformed with RACE PCR products at least 10 single colonies were picked from each plate. To analyze their genetic make-up plasmid DNA was isolated, amplified via M13-PCR and sequenced.

Sequencing was performed from both sides as ligation of the PCR product could happen either way.

4.1.2.1 Sequencing analysis reveals the partner gene of ETV6

The obtained sequences were fed into the BLAST software. The sequence of the ETV6 transcript was found in most cases from either side. This makes sense as normal (non-translocated) ETV6 loci were also present in the original sample. Also, parts of the chromosome 12 genomic sequence were obtained as BLAST result with all of them containing part of the ETV6 locus. A few results included sequences of the ETV6 transcript followed by 34 nucleotides of chromosome X that were separated from each other by a variable number of nucleotides. In one case part of the TK1 (thymidine kinase 1) transcript (chromosome 17) was the result of both directions. This was some

kind of artifact as the translocation between chromosomes 12 and 17 could not be proved by subsequent whole chromosome paints. In another case the NTRK3 transcript (neurotrophin tyrosine receptor kinase, type 3), also referred to as TrkC, was found on the forward sequencing reaction containing the polyA tail of the transcript, whereas the reverse reaction identified ETV6 as 5´ part of the transcript. This latter finding was of great interest to this project as NTRK3 is located to chromosome 15 which is acrocentric as proposed for the unidentified chromosome involved in the translocation with chromosome 12.

As far as the originally identified translocation t(10;12) is concerned, this genetic rearrangement was not detected on the level of RNA. A statistical overview of the sequencing results is shown below (figure 4.4).

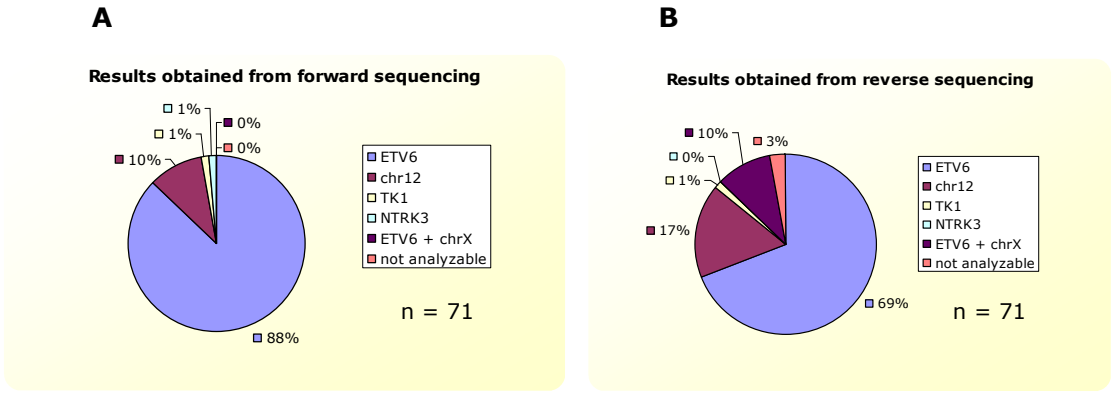

Figure 4.4 A, B *Statistical view of sequencing results. These two diagrams show the percental distribution of sequencing results in forward reaction (A) and reverse reaction (B).*

4.1.3 Verification of the translocation t(12;15)

Whole Chromosome Paints were performed to verify the translocation involving chromosome 15, and thus to augment the probability that the gene NTRK3 on chromosome 15 is actually fused to ETV6 on chromosome 12 (see figure 4.5).

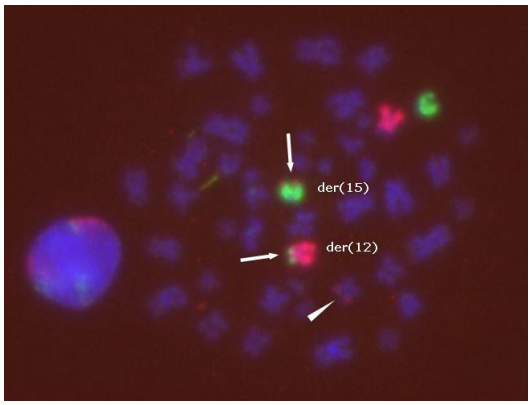

Figure 4.5 *WCP of chromosomes 12 and 15. Chromosome 12 is painted red and chromosome 15 painted green. The arrows indicate the reciprocal translocation giving rise to derivate chromosomes der(12) and der(15). The arrow head marks the additional chromosome 12 translocation.*

After proving the existence of this translocation further studies concerning the resulting fusion transcript were done.

4.1.4 NTRK3, the ETV6 partner

NTRK3 is the receptor for a neurotrophin called NT-3. Upon interaction signaling cascades are activated that regulate development and maintenance of the vertebrate nervous system. NT-3 is widely distributed in neural as well as in non-neural tissues and its receptor NTRK3 shows expression in both neural tissues as well as in hematopoietic and epithelial cells [47].

The NTRK3 gene or transcript is organized as follows: exon 1 contains the 5´ UTR and encodes the signal peptide. Exons 2 through 9 encode diverse domains, such as two cystein clusters, one leucine-rich motif and two Ig-like domains. The transmembrane domain is encoded by exons 10 and 11, and the juxtamembrane domain by exons 11 through 13, while the tyrosine kinase domain is encoded by exons 13 through 18.

The non-catalytic isoform of NTRK3 exhibits exons 13b and 14b as its final two exons which code for an alternative intracellular domain of 83 residues rather than the consensus tyrosine kinase domain [48]. This truncated splice variant is also referred to as isoform B [49]. The NTRK3 genomic and protein structures are shown below (figure 4.6).

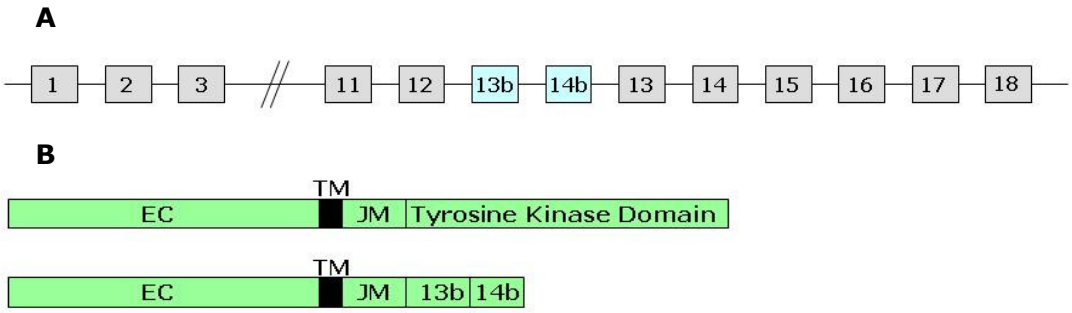

Figure 4.6 A, B *Schematic representations of NTRK3. The genomic organization is shown in A and the NTRK3 peptide isoforms in B. In A the final two coding exons of the truncated isoform are highlighted blue and for spatial reasons exons 4-10 are omitted; in B the tyrosine kinase domain is encoded by exons 13-18. EC, extracellular domain; TM, transmembrane domain; JM, juxtamembrane domain. (Nomenclature of the exons is adopted from [47]).*

The truncated NTRK3 proteins are mainly thought to act as dominant negative molecules that inhibit signaling of their catalytically active counterparts [50]. Truncated NTRK3 was also shown to trigger a signaling pathway leading to membrane ruffling and the formation of cellular protrusions [51]. Moreover, mouse models suggest that the non-catalytic NTRK3 plays an indispensable role in development [52]. However, its exact physiological function remains elusive.

Alternative splicing can result in two additional isoforms called C and D, which arise from in-frame deletion of exon 9 (27 nucleotides) and exon 16 (42 nucleotides), resp. in regard to the canonical isoform A depicted in figure 4.6 B [49]. The isoforms C and D are not further discussed as they were of no relevance in this diploma thesis.

4.1.5 Characterization of the ETV6-NTRK3 fusion transcript

After verification of the translocation the fusion transcript was tried to be specified.

4.1.5.1 RT-PCR proves alternative splicing of fusion transcript

In order to investigate expression of the chimeric gene on the level of RNA reverse transcriptase PCRs were done.

As the initially found sequence represented the truncated version of the neurotrophin receptor kinase the idea that other isoforms could be found as ETV6 partners, too, sounded reasonable. This was tested in nested RT-PCR experiments.

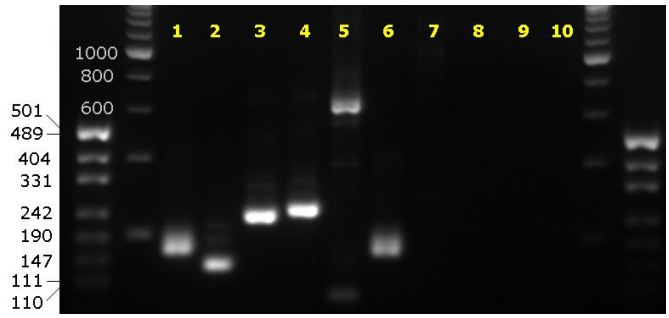

Figure 4.7 *Nested RT-PCR. Results indicate that ETV6 builds fusion transcripts with both the truncated and catalytically active NTRK3 isoforms.*

PCR products are shown in figure 4.7. As positive control patient A cDNA was amplified with ABL Iii and ABL IIIi primers specific for the widely distributed c-abl receptor tyrosine kinase gene (lane 1, 173 bp).

In lane 2 the fusion transcript consisting of ETV6 and truncated NTRK3 is clearly detectable (142 bp; ETV6exon5 and NTRK3SequF, the latter located in NTRK3 exon 13b).

Lanes 3, 4 and 5 show transcripts of ETV6 fused to the catalytically active NTRK3 isoforms (245 bp, 260 bp and 678 bp, resp.; ETV6exon5 and NTRK3cod.exon14I or NTRK3cod.exon14II, or NTRK3cod.exon17 primers, resp., all NTRK3 primers specific for the tyrosine kinase domain).

No signal is obtained for the reciprocal translocation (NTRK3-ETV6) in lanes 8 and 9 where cDNA was tried to be amplified with NTRK3cod.ex12fw1 and ETV6cod.ex6rev, and NTRK3cod.ex12fw and ETV6cod.ex6rev primer sets, respectively.

Two different negative controls were run in the PCR experiment. First, cDNA from a different patient not showing the translocation in question was tried to be amplified by use of ETV6exon5 and NTRK3SequF primers (lane 7). Thereby, the fusion was shown to be specific for patient A as it was not detected in the other patient. In order to eliminate the possibility of a non-functional cDNA template RT-PCR using ABL Iii and ABL IIIi primers was run with this other patient's cDNA (lane 6).

Second, ddH_2O was used in the PCR alongside ETV6exon5 and NTRK3SequF primers (lane 10).

4.1.5.2 Identification of coding exons participating in the chimeric transcript

Fusion transcripts were more closely characterized by sequencing of RT-PCR products to get an insight into the structure of resulting chimeric transcript and its constituting exons.

Sequencing definitely confirmed the expression of the ETV6-NTRK3 fusion gene in patient A. Both the truncated and the catalytically active isoform were verified to be fused to ETV6 (see figure 4.8). They were both shown to be in-frame fusion partners of ETV6.

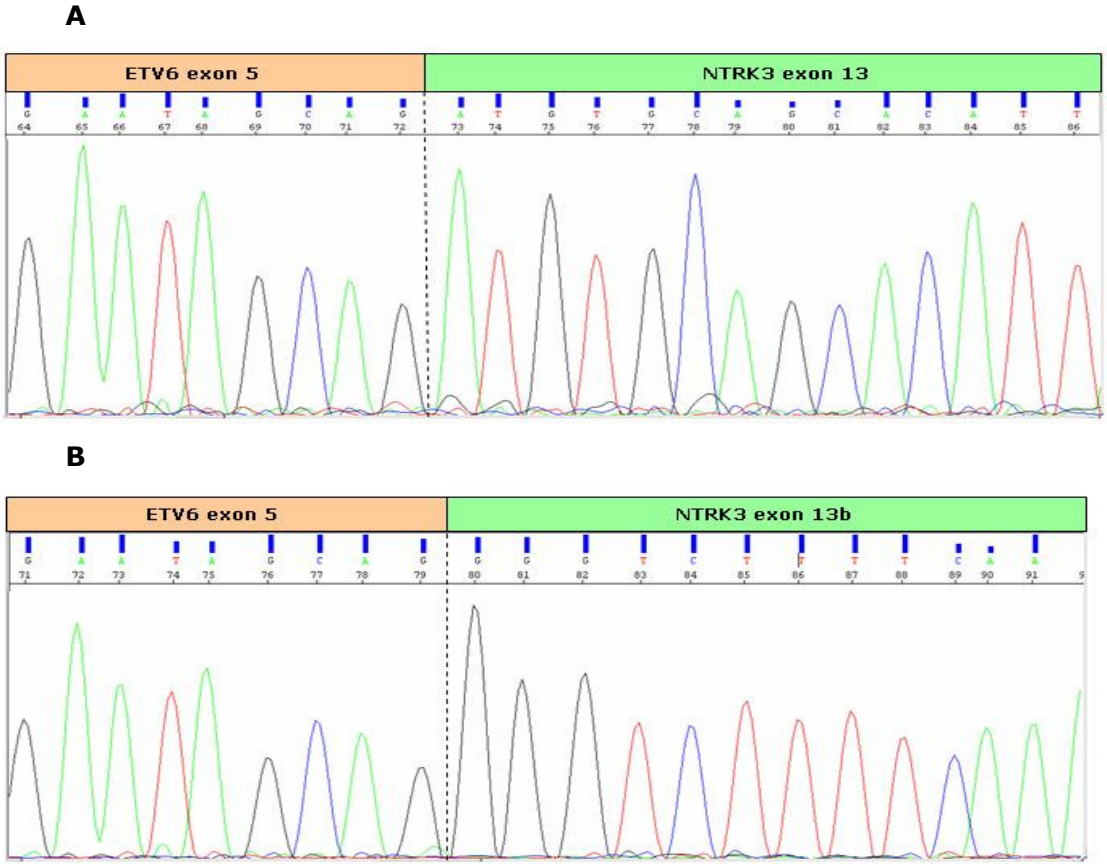

Figure 4.8 A, B *Sequences of fusion transcripts. Electropherogram shows the fusion transcripts involving ETV6 and the catalytically active NTRK3 isoform (A) and ETV6 fused to the truncated NTRK3 isoform (B). In either case, sequencing was done in both directions with only one being shown. Primer ETV6SequRneuNstd used in A, primer ETV6SequRneu used in B.*

The fusion transcripts are depicted below with ETV6 colored pale orange, and NTRK3 colored green.

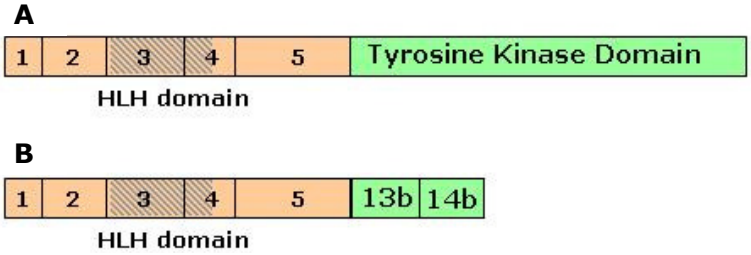

Figure 4.9 A, B *Proposed structure of the entire fusion transcripts. ETV6 being fused to the catalytically active NTRK3 isoform in (A) and to the truncated isoform of NTRK3 in (B).*

4.2 Mutational analysis of genes frequently involved in AML

The frequently mutated NPM1 and FLT3 genes in AML were tested for patient A in order to have a more complete patient profile.

4.2.1 Analysis of the NPM1 transcript sequence

Sequencing analysis of the NPM1 exon 12 allows detection of a frequent mutation altering the amino acid tryptophan at position 288 resulting in the pathological outcome.

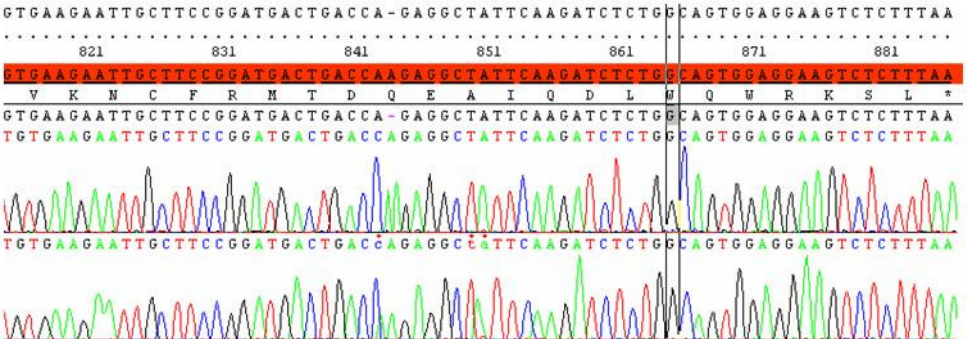

Figure 4.10 *Electropherogram of NPM1 sequencing. Reference NPM1 transcript (upper sequence) is shown in comparison to the patient NPM1 transcript (lower sequence).*

Figure 4.10 indicates that sequencing analysis of the patient NPM1 transcript did not detect any mutation leading to alteration of the tryptophan residue as marked by the cursor position.

4.2.2 Analysis of the FLT3 genomic sequence

The FLT3 gene is tested for the existence of internal tandem duplications using DHPLC. In case of a mutation more than one fragment is amplified during the PCR reaction. Thus, further peaks will be obtained in addition to the wild type signal.

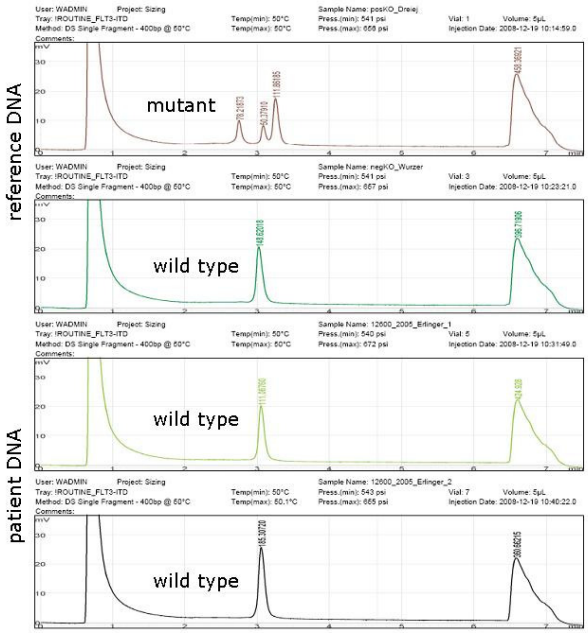

Figure 4.11 *Chromatogram of FLT3 analysis. Peak profiles of reference DNA in comparison to patient DNA are depicted.*

As illustrated by the above chromatogram (figure 4.11), patient DNA turned out not to be mutated since single peaks were obtained in DHPLC analysis.

4.3 Implementation of the BAC FISH method

After completion of a great deal of bureaucracy the import license for *E.coli* harboring BACs issued by the Ministry of Health, Family, and Youth was obtained for our laboratory which is valid through June 2009.

After this absolutely necessary step, appropriate BAC clones were selected for downstream experimental procedures. Two different freely accessible data bases on the internet were used to search for the BAC clones of interest. On the one hand, the University of California, Santa Cruz genome browser (http://genome.ucsc.edu/index.html), Assembly March 2006, and on the other hand the BAC/PAC collection data base of the University of Bari, Italy (http://www.biologia.uniba.it/rmc/) were used for identification of useful BAC clones. Selected BACs were then ordered at the BACPAC Resources Center at Children's Hospital Oakland Research Institute (CHORI) in California, USA and subsequently labeled by nick translation. These self made DNA probes were then used on non-pathological patient material to check their specificity and were subsequently hybridized on patient material of interest to fulfill their purpose in breakpoint specification.

4.4 Patient B: Searching for the breakpoint

According to conventional cytogenetics the patient carried the translocation t(9;21). Chromosome bands 9q13 and 21q22 seemed to be involved.

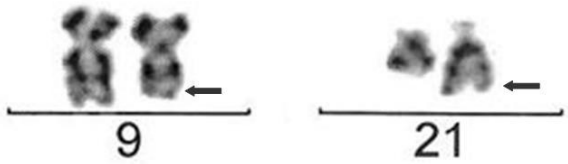

Figure 4.12 *Translocation t(9;21). Chromosomes 9 and 21 showing structural abnormalities (arrows) due to the translocation between them.*

The translocation t(9;21) was seen in 12 out of 31 metaphases in routine cytogenetics.

To further narrow the area of a possible breakpoint, BACs were ordered according to chromosome information available on the University of California, Santa Cruz Genome Browser.
As the band 9q13 can be easily covered by only three different BACs, RP11-2M11, RP11-151G22 and RP11-825L14, these were ordered, labeled and used in FISH experiments. If the breakpoint actually happened somewhere within this particular band a split signal of any two of these BACs will be detectable.
For illustrative reasons the localization of the BACs relative to the band 9q13, to the genes and to each other is shown in the figure below.

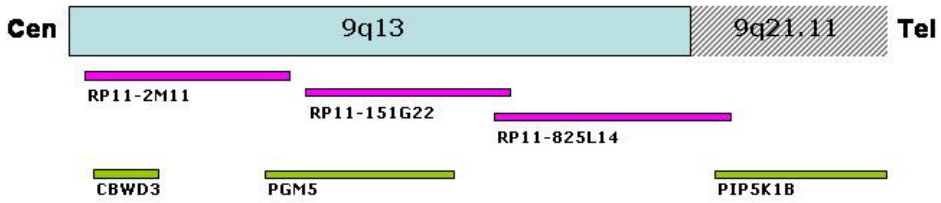

Figure 4.13 *Schematic illustration of band 9q13 (500 kb in size). Part of band 9q21.11 and of the assigned BAC clones (magenta) relative to the genes (green) are also shown. Not drawn to scale; centromeric to telomeric direction as indicated.*

Two rounds of BAC hybridization were carried out. Since the first one using RP11-2M11 and RP11-151G22 did not result in a split signal (see figure 4.14 A), hybridization with RP11-2M11 and RP11-825L14 was performed (see figure 4.14 B), which again delivered fusion signals only.

The BAC RP11-2M11 turned out to show multiple signals on chromosome 9 and an additional hybridization signal close to the chromosome 2 centromer (see figure 4.14 A and B). Keeping the additional specificity in mind it did not interfere at all with the results and validity of the FISH studies.

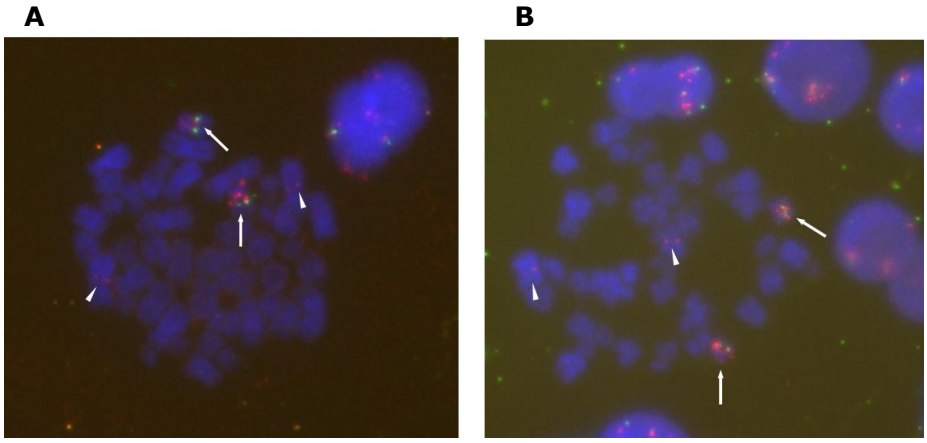

Figure 4.14 A, B *FISH done with 9q13 specific BAC probes. The probes RP11-2M11 (red) and RP11-151G22 (green) were applied in the FISH experiment shown in A, whereas RP11-2M11 (red) and RP11-825L14 (green) were used for hybridization in B. In each panel, arrows indicate the fusion signal, arrow heads mark the additional specificity of the probe RP11-2M11 for chromosome 2.*

Despite our expectations no split signal was detectable. Instead, both approaches delivered fusion signals on chromosome 9 indicating the breakpoint must occur outside the assumed band 9q13.

4.5 Patient C: Narrowing of the breakpoint and proposal for a candidate gene

Conventional cytogenetics revealed a translocation involving chromosomes 4 and 13. The translocation seemed to be located just underneath the chromosome 13 centromer and was therefore designated t(4;13)(q35;q12).

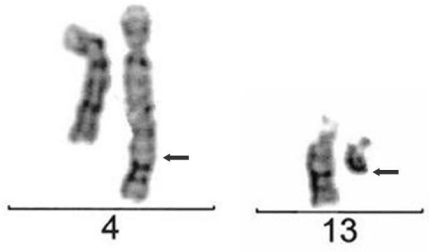

Figure 4.15 *Translocation t(4;13). Chromosomes 4 and 13 showing clear karyotypic abnormalities (arrows) due to the translocation.*

In routine cytogenetics the translocation was found to be present in virtually 100% of the examined meta- and interphases.

As the breakpoint on chromosome 13 was assumed to be very close to the centromer, BAC clones situated in this region were ordered, labeled and used to walk along the chromosome until a split signal between two of them appeared. After a few rounds of BAC hybridization the breakpoint could be constrained to a region of about 497 kb that was flanked by the clones RP11-933M24 and RP11-523H24 (see figure 4.16).

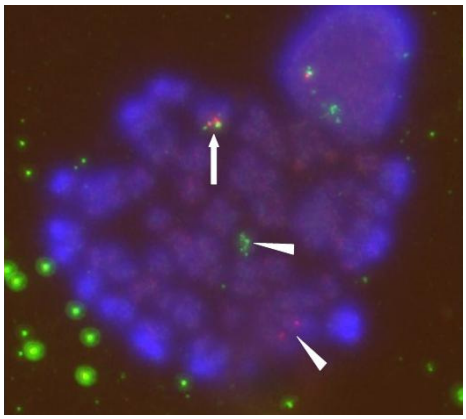

Figure 4.16 *Hybridization of RP11-933M24 (green) and RP11-523H24 (red). The arrow marks the fusion signal on non-rearranged chromosome 13. Arrow heads indicate the split signal due to the breakpoint between the probes.*

Within this 497 kb region there is a candidate gene, TPTE[6]2, located. In order to find out if this gene might be affected by the translocation, the breakpoint was further approximated by two additional BAC clones, namely RP11-134H3 and RP11-1031I2. Figure 4.17 gives a schematic overview of the experiment.

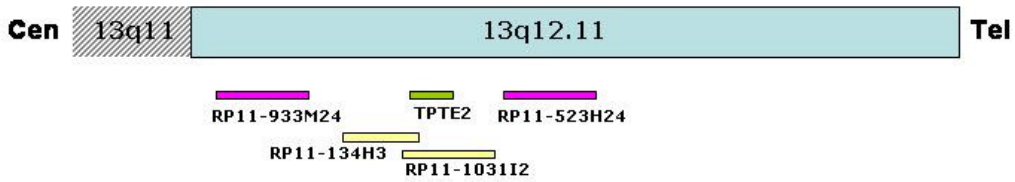

Figure 4.17 *Schematic representation of the band 13q12.11 (3,8 mb in size). BAC clones narrowing the breakpoint to 497 kb are colored magenta, the candidate gene probably involved in the translocation (TPTE2) is colored green, and additional BAC clones for further approximation of the breakpoint are colored yellow. Centromeric to telomeric direction as indicated.*

The figure below (figure 4.18) shows the visualization of the RP11-134H3 and RP11-1031I2 probes which were used in order to further approximate the breakpoint.

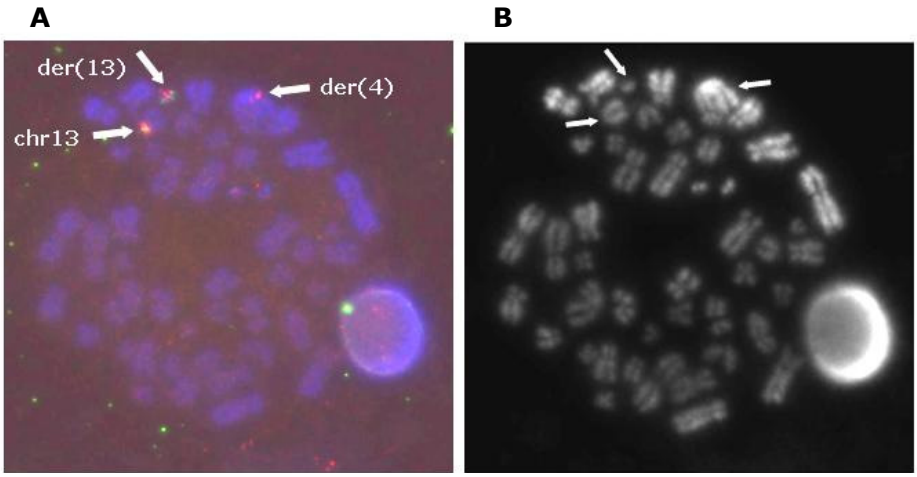

Figure 4.18 A,B *FISH performed with 13q12.11 specific probes. RP11-134H3 (green) and RP11-1031I2 (red) were applied. In panel A, arrows mark the fusion signals on normal and pathologic chromosome 13 as well as the split signal on chromosome 4 (sole red signal). In panel B, chromosomes are shown as visualized in the DAPI filter for better visibility of their structural appearance. Arrows mark the chromosomes of interest.*

As clearly visible in the above figure (figure 4.18), hybridization of these two probes definitely showed an additional red signal on the long arm of derivative chromosome 4, der(4), which folds back on itself (clearly visible in panel B).
For clarity the signal pattern is schematically illustrated below (figure 4.19).

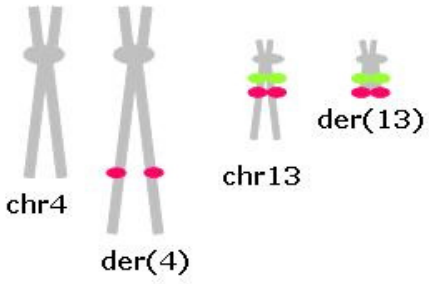

Figure 4.19 *Illustration of the signal pattern. The probes RP11-134H3 (green) and RP11-1031I2 (red) deliver 2 fusion signals representing chromosomes 13 and a sole red signal representing the derivative chromosome 4.*

The obtained signal pattern demonstrates that the breakpoint is located within the region of RP11-1031I2 (red). This increases the possibility that the translocation actually involves the candidate gene TPTE2.

5 Discussion

The identification of fusion genes and their role in aberrant cellular functions are of very great importance in disease and drug research.
The function of chimeric molecules needs to be elucidated so that therapy can be specifically developed. In case of constitutively active kinases, small inhibitors have been artificially synthesized that specifically inhibit aberrant enzymatic activity and therefore terminate cellular processes like for instance proliferation. In some cases inhibitors even show cross-reactive effects, i.e. they act on fusion products they were not designed for, e.g. imatinib, originally developed as CML therapy, succeeds in treatment of FIP1L1-PDGFRα positive eosinophilia patients.
The very first step in the process of drug development is the identification of a disease's origin, e.g. the existence of fusion genes. As soon as the exact structure and the role in signaling or other processes are known, specific small molecules will be designed that act on these pathogenic cellular components. Of course, this whole process is of very long duration and is absolutely based on the prior identification of such aberrantly acting molecules.

5.1 Patient A's genetic make up

5.1.1 FLT3 and NMP1 mutational status

FLT3 and NPM1 were shown not to be mutated, results that were consistent with our expectations. Usually, both FLT3 and NPM1 show genetic alteration in up to 33% and 60%, resp. of cytogenetically normal patients. This patient, though, showed clear chromosomal rearrangements and was therefore not expected to be tested positive for these molecularly defined mutations. However, we decided to examine their genetic status for the sake of a more complete patient profile on possible AML mutations.

5.1.2 The ETV6 fusion partner

When sequencing analysis of cloned RACE PCR products was performed one expected to detect the ETV6 transcript sequence from the 5´ end, whereas the 3´ polyA end was supposed to be part of the fusion partner.
It was obvious that the ETV6 sequence would also be detected from both ends as the translocation was not found in a hundred percent of the patient's cells and the normal ETV6 locus was still present.
So in most of the sequencing reactions ETV6 was found from either direction. Many other results constituted chromosome 12 genomic sequences, yet all of them contained part of the ETV6 locus. These results were regarded as worthless in the search for fusion partners.
Furthermore, some ETV6 transcript sequences were found to be followed by an inconsistent number of nucleotides that were themselves followed by a short, non-variable span of the chromosome X genomic sequence. These cases were not further investigated as no physiological role was attributable to this kind of genomic constellation.
Another sequencing reaction detected the thymidine kinase 1, TK1, transcript from either direction. The TK1 gene is located on chromosome 17 and therefore the patient's material was tested for the translocation t(12;17) by WCP assays. As expected, this sequencing reaction was some kind of artifact because the translocation was not verified by whole chromosome paints. Besides, the fact that TK1 was sequenced from either direction indicated that this was some kind of doubtful result.
One other sequencing result detected ETV6 at the 5´end and the NTRK3 transcript sequence at the 3´ polyA containing end. This attracted a great deal of attention as the NTRK3 locus is found on chromosome 15 which has an acrocentric shape. This was concordant with the unidentified additional chromosome found to be translocated to chromosome 12.
As far as the initially identified t(10;12) is concerned this rearrangement was not detected on the level of RNA. Hence, it seems not to be expressed.

5.1.3 ETV6-NTRK3 fusion and its underlying t(12;15)

Whole chromosome paints confirmed the underlying translocation t(12;15) of the ETV6-NTRK3 fusion (see figure 4.5). This translocation was not detected by routine chromosome banding techniques because it is too cryptic. The regions exchanged between chromosome 12 and 15 are very similar in size and banding characteristics. Therefore, the translocation

is easily overlooked in conventional cytogenetics. Even though the reciprocal translocation occurred on the genomic level it was not detectable on the level of RNA by RT-PCR suggesting it is not expressed.
In figure 4.5 another translocation involving chromosome 12 is visualized, most probably the initially found translocation t(10;12) which could not be detected on the level of RNA by RACE PCR and sequencing. In order to rule out the possibility of a complex translocation involving chromosomes 10, 12 and 15, t(10;12;15), whole chromosome paints were performed. The visualization of chromosomes 10 and 15 did not show any translocation signals (data not shown). Therefore, a complex translocation was excluded.

5.1.4 The ETV6-NTRK3 fusion transcript

The existence of the translocation t(12;15) was verified on the genomic level by whole chromosome paints, and in order to investigate the expression of the fusion genes RT-PCR analysis was performed.
As the NTRK3 transcript that was initially detected by sequencing turned out to represent a truncated, non-enzymatic isoform, the existence of further NTRK3 isoforms in the fusion transcript suggested itself.
Even though RT-PCR could not detect the reciprocal NTRK3-ETV6 fusion transcript, it succeeded in showing the expression of the originally detected fusion transcript on one hand, as well as the expression of the catalytically active NTRK3 isoform as part of the fusion on the other hand. In either case primer sets were used that were located in exons directly flanking the breakpoint.
For definite evidence, RT-PCR amplification products were sequenced with appropriate primers (see Figure 4.8). Based on these results, the proposed entire fusion transcripts contain ETV6 exons 1 through 5 fused to NTRK3 exons 13b and 14b, or NTRK3 exons 13 through 18, resp. (see Figure 4.9).
So far, the catalytically active ETV6-NTRK3 fusion proteins have been detected in a significant fraction of non-hematopoietic tumors, e.g. secretory breast carcinoma [20], congenital fibrosarcoma [53] or mesoblastic nephroma [54]. Their transforming ability is based on constitutive activation of the NTRK3 moiety with downstream activation of Ras-MAP kinase and phosphatidyl inositol-3-kinase (PI3K)-AKT pathways [20].
Interestingly, the ETV6-NTRK3 fusion has been reported in only one case of hematological malignancy, i.e. an AML (FAB M2) [19]. The reported case in this diploma thesis is an AML, too, but M0 according to the FAB classification. Furthermore, there are two clear differences between the previously detected hematological ETV6-NTRK3 fusion and the

one described here. First, the fusion transcript identified in this study exhibits ETV6 exons 1 through 5 in contrast to the previously published case which contained only the first four ETV6 exons. Therefore, the entire central domain, made up partly of exon 4 and the entire exon 5, is still present and might therefore enable further protein-protein interactions. The second difference is that ETV6 was only shown to be fused to the catalytically active isoform in the earlier report. In our case we demonstrated that alternative splicing of the transcribed ETV6-NTRK3 gene takes place and gives rise to the truncated NTRK3 isoform which is known to induce signaling that results in membrane ruffling and cellular protrusions [51], structural changes that support cell migration.

Unfortunately, insufficient patient material did not allow the investigation of ETV6-NTRK3 expression on protein level. The oncogenic activity of both variants remains to be revealed. Thereby one must keep in mind that fusion products containing ETV6 have been shown to involve diversely regulated aberrant mechanisms. As far as kinase domain proteins are concerned, ETV6 influences the fusion partner's activity via its HLH (dimerization) domain [22]. Regarding the ETV6-NTRK3 fusion, data prove that the NTRK3 kinase domain by itself does not provoke transformation activity. More precisely, it was demonstrated that NIH3T3 cells cannot be successfully transformed by ETV6-NTRK3 chimeric proteins that still possess a perfectly operating NTRK3 kinase domain but lack a functional ETV6 HLH domain [55]. In other cases, the ETV6 promotor driving the transcription of the fusion gene is thought to confer oncogenic potential [22]. It is possible that the two variants of fusion products identified in this case may bear different potentials in transforming actions.

5.1.5 Suggestions for continuation

As mentioned earlier, it was not possible to investigate the fusion on protein level due to insufficient patient material. Although cells were available, there was not much material left because of the preceding FISH studies. Additionally, these cells were stored in fixative making them most probably useless for protein extraction due to denaturing conditions and cross-linking procedures during fixation.

However, one can try to isolate the entire fusion transcript by RT-PCR, clone it into an expression vector and have it expressed in vast amounts. In subsequent interaction studies, e.g. yeast-2-hybrid systems, binding partners could be searched for to get an insight into possible signaling pathways. Also, a myeloid, stably transfected cell line could be used to investigate the fusion product's function. One could examine its influence on proliferation, its phosphorylation status or its migration behavior towards chemokines, etc.

5.2 Patient B's untraceable breakpoint at 9q13

According to conventional cytogenetic data the translocation was assumed to involve bands 9q13 and 21q22 and was therefore defined as t(9;21)(q13;q22). The chromosomal band 9q13 turned out to be only ~ 500 kb in size and could easily be covered by three different BAC clones. When hybridizing these specific probes to the area of interest no breakpoint could be located in this region.

This incidence shows that the correct interpretation of karyograms is very challenging, especially when the patient's metaphases are of poor quality or when the chromosomal areas surrounding the band of interest are very similar in size and appearance. Moreover, cytogenetics represent a diagnostic approach that differs greatly among patients and frequently shows differences in chromosomal structures of individual patients even without the existence of pathological chromosomal aberrations.

5.2.1 Suggestion for alternative approaches

The exact determination of the breakpoint requires additional analyses. One possible method is using BAC-FISH to walk along the affected chromosome arm and get an insight into the relative location of the breakpoint. The greater the target area is in size the more rounds of hybridization assays are required and thus, a considerable amount of patient material will be needed. An alternative to this cost-effective but material consuming method would be the usage of multicolor chromosome banding kits (MetaSystems, Germany). These differently labeled, region-specific paints represent a very good method to locate chromosomal rearrangements. Even though this latter approach is less time and material consuming, multicolor chromosome banding paints with sufficient resolution are not available for every arbitrary chromosomal region.

5.3 Detection of a candidate gene for patient C

The translocation t(4;13) involved the band 13q12.11 which is almost 4 mb in size. With the help of BAC probes this region was successfully narrowed down to a smaller area of only 497 kb. A candidate gene within this chromosomal band could also be identified, TPTE2.

5.3.1 TPTE2 – a tumor suppressor phosphatase?

TPTE2, or alternatively called TPIP[7] [56], is a phosphatase of the PTEN[8] tumor suppressor family [57]. Several splice variants exist, two of which seem to be functionally different. TPIPα is localized to the endoplasmic reticulum membrane and possesses phosphatase activity to metabolize phosphatidylinositol 3,4,5-trisphosphate. By contrast, TPIPβ lacks phosphatase acitivity and is found in the cytoplasm [58]. TPIPα might represent a tumor suppressor and functional homologue of PTEN which inhibits cell cycle progression, cell survival and motility by antagonizing PI3-kinase activity via dephosphorylation of phosphatidylinositol 3,4,5-trisphosphate [59].

5.3.2 TPIP, a proposed candidate but not definitely translocated

When TPIP was identified as candidate gene no definite evidence for actual translocation could be provided, though. The region between the BAC probes RP11-933M24 and RP11-523H24 was demonstrated to include the breakpoint (see figure 4.16). Two more BACs were then applied as probes, one of which, RP11-1031I2 (red), spanned the whole TPTE2 gene reaching into the non-coding area telomeric to TPTE2. An additional BAC probe, RP11-134H3 (green), was hybridized which was located centromeric to the candidate gene and reached into it (see figure 4.17). The idea was that if the breakpoint occurs very close to the centromeric end of the gene, a hybridization signal probably won't arise in case the complementary regions between the gene and RP11-1031I2 were too short. That is why the green RP11-134H3 was hybridized, too.

According to the signal pattern obtained (2 fusion signals, 1 red signal), the breakpoint is definitely located downstream of the RP11-134H3's telomeric end and within RP11-1031I2. This is because the 2 fusion signals represent the normal chromosome 13 as well as the shortened version of chromosome 13 emerging from the translocation. The sole red signal is therefore located to the derivative chromosome 4 representing the second half of the broken RP11-1031I2.

The only question that still remains now is if this probe's signal is broken within the coding region of the TPIP gene or if the breakpoint is located even closer to the telomere and thus within non-coding areas of chromosome 13. The length of the non-coding region complementary to the RP11-1031I2 probe is 67 kb in size. It is possible that the

[7] TPTE and PTEN homologous inositol lipid phosphatase

[8] Phosphatase and tensin homologue deleted on chromosome 10

breakpoint occurs in this non-coding area so that the probe would still be able to hybridize. In order to continue on this issue, definite proof needs to be provided whether or not TPTE2 is affected by the translocation.

5.3.3 Suggestions for continuation

In order to definitely identify or exclude TPTE2 as translocated gene, further BAC hybridization assays could be performed. In case of confirmation, its chromosome 4 fusion partner needs to be detected. This can be done by RACE PCR again which turned out to be a nice and reliable method in this matter as is shown for patient A. Designing an appropriate RACE PCR can be challenging, though, in this case as TPTE2 seems to exist in multiply expressed isoforms. Thus, the RACE PCR probably results in many different bands representing various isoforms in the fusion transcript.

5.4 BACs – suitable tools in routine?

The usage of BACs as DNA probes was successfully established and they were effectively applied in fluorescence in-situ hybridizations. Moreover, they represent a more cost-efficient method compared to commercially available, ready-to-use DNA probes.
Despite the fact that it takes about 14 working days from the day of BAC order to the day of visualization in the fluorescence microscope, BAC FISH represents a very nice and suitable method for routine diagnostics.
Routine is restricted to tests that are run frequently. Thus, BACs needed for this purpose have to be ordered once and made ready for labeling. These fluorescently labeled probes are made fast and can be re-made from the glycerol stocks of specific BAC clones virtually an unlimited number of times.
The application of BAC FISH in routine diagnostic is of considerably great importance since purchasable probes are not available for all of the diagnostically interesting chromosomal breakpoints, including rearrangements considered in the WHO classifications of tumors.
Therefore, BAC FISH represents an important and efficient as well as considerably low-cost method for diagnostic purposes.

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A. Appendix

A.1 Primers

Primers marked with (1) one asterisk (*) were ordered at Metabion international AG, (2) two asterisks (**) were ordered at TIB Molbiol, (3) three asterisks (***) were ordered at genXpress, and all other primers were ordered at Applied Biosystems. Primers are listed in alphabetical order.

Table A.1.1 Primers (5´→ 3´) used for RACE PCR

Name	***Sequence***
ETV6A	CAT ATA CAC CTC CAG AGA GCC CAG TGC
ETV6Anested	CTT CAT GTT CCA GTG CCT CGA GCG C
ETV6B	CTG AAG CAG AGG AAA CCT CGG ATT C
ETV6Bnested	CAC CCT GGA AAC TCT ATA CAC ACA CAG C
ETV6C	CAT GCC CAT TGG GAG AAT AGC AGA CTG
ETV6Cnested	CTG ACA GCC GGT ACG AAA ACT TCA TCC

Table A.1.2 Primers (5´→ 3´) used for PCR experiments

Name	***Sequence***
ABL IIi**	GCC TCA GGG TCT GAG TGA AGC CGC TCG TTG
ABL IIIi**	TGT GAT TAT AGC CTA AGA CCC GGA GCT TTT C
ABL IIIo**	CCA TTT TTG GTT TGG GCT TCA CAC CAT TCC
ABL IIo***	CAG CGG CCA GTA GCA TCT GAC TTT G
ETV6cod.ex6rev*	GAT TCT TTG TCC TCC CAT CG
ETV6cod.ex8rev*	GCC ACT CAT GAT TTC ATC TG
ETV6exon5*	ATG CCC ATT GGG AGA ATA GC
ETV6SequRneu	CCC ATC AAC CTC TCT CAT CG
ETV6SequRneuNstd*	CTC TCT CAT CGG GAA GAC C
ITD F*	GCA ATT TAG GTA TGA AAG CCA GC
ITD R*	CTT TCA GCA TTT TGA CGG CAA CC
M13F	TGT AAA ACG ACG GCC AGT
M13R	CAG GAA ACA GCT ATG ACC
NPM1b-for	GGT TCT CTT CCC AAA GTG G
NPM1b-rev	GGC ATT TTG GAC AAC ACA TTC
NTRK3cod.ex11fw*	CCT GTT GGT GGT TCT CTT CG
NTRK3cod.ex12fw*	GGA CAC AAC TGC CAC AAG C
NTRK3cod.ex12fw1*	CAT CCC TGT CAT TGA GAA CC
NTRK3cod.exon14I*	AAC TTG ACA ATG TGC TCA TGC
NTRK3cod.exon14II*	CCG CAC ACT CCA TAG AAC

NTRK3cod.exon17*	GAA CTT CCG GTA CAT GAT GC
NTRK3PTKisoA,C,D	CCT CCG TGT TTG AGA GTT G
NTRK3SequF	AAG ACA CTT CCC CAC TCT GG

Table A.1.3 Primers (5´→ 3´) used for sequencing

Name	***Sequence***
ETV6SequRneu	CCC ATC AAC CTC TCT CAT CG
ETV6SequRneuNstd	CTC TCT CAT CGG GAA GAC C
M13F	TGT AAA ACG ACG GCC AGT
M13R	CAG GAA ACA GCT ATG ACC
NPM1b-F	GGT TCT CTT CCC AAA GTG G
NPM1b-R	GGC ATT TTG GAC AAC ACA TTC
NTRK3cod.exon14I	AAC TTG ACA ATG TGC TCA TGC
NTRK3cod.exon14II	CCG CAC ACT CCA TAG AAC
NTRK3SequF	AAG ACA CTT CCC CAC TCT GG
SP6	ATT TAG GTG ACA CTA TAG
T7	TAA TAC GAC TCA CTA TAG GG

A.2 DNA markers

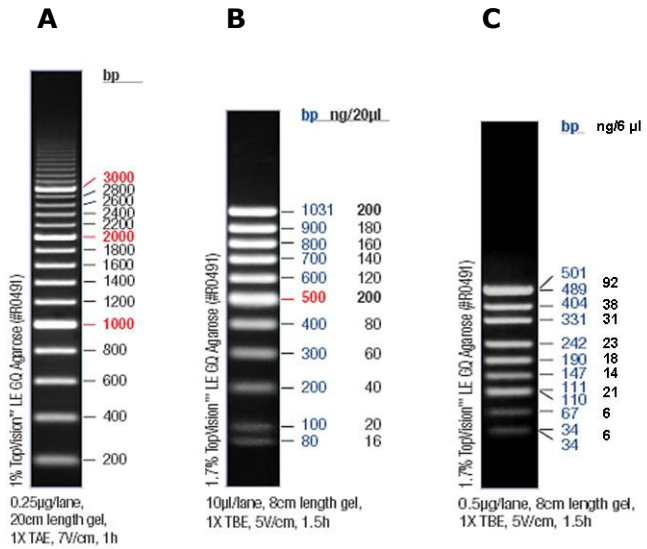

Figure A.2.1 A, B, C *DNA markers used for agarose gel electrophoresis. O'RangeRuler™ 200 bp DNA Ladder in A, MassRuler™ DNA Ladder, Low Range in B, pUC19 Hpa II DNA Marker in C (pictures adopted and slightly modified from [60]).*

A.3 Vectors

The vectors below were either used for cloning procedures within the RACE experiment or they harbored the genomic fragment of interest which were ordered as BAC clones from the BACPAC Resources Center, Oakland, California.

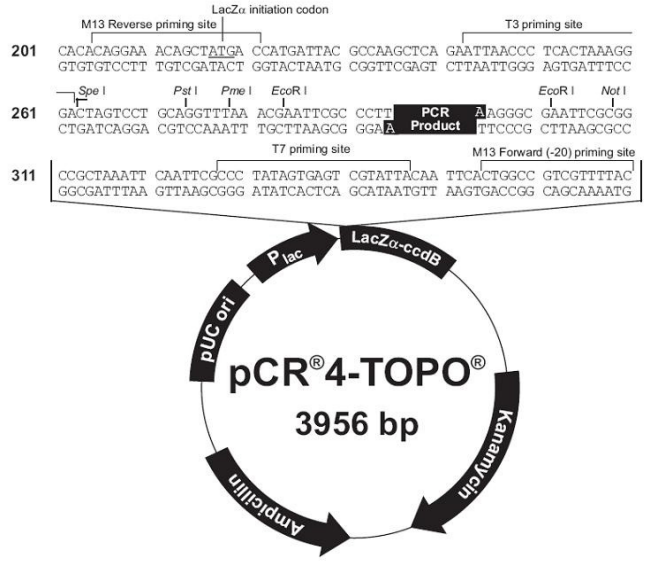

Figure A.3.1 *pCR®4-TOPO® vector used for cloning of RACE PCR products (figure adopted from [38]).*

A **B**

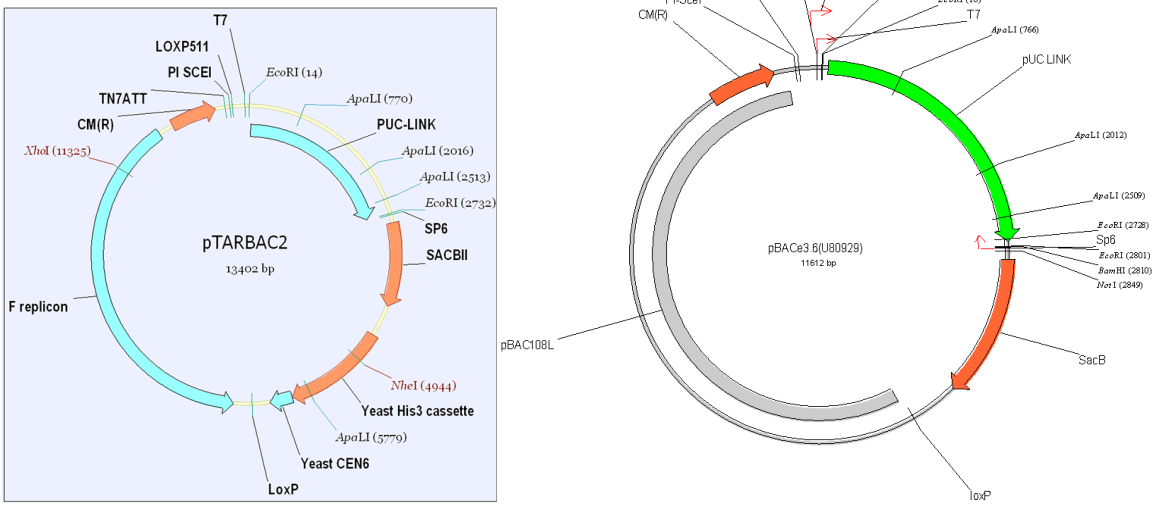

Figure A.3.2 A, B *BAC clones that were ordered as* E.coli *LB stabs at BACPAC Resources are constructed either in pBACe3.6 or pTARBAC2 vectors (pictures from [61]).*

A.4 BAC clones

Table A.4.1 BAC clones used in FISH experiments.

Name	*Chromosome*	*Chromosomal localization*	*Specificity*
RP11-64I8	13q12.3	30 406 381 – 30 571 172	ensured
RP11-910O20	13q12.3	29 971 980 – 30 126 905	ensured
RP11-764B5	13q12.3	27 809 291 – 27 962 431	ensured
RP11-367D4	13q12.13	25 764 973 – 25 929 701	ensured
RP11-309I15*	13q12.12	23 481 640 – 23 597 770	ensured
RP11-149B7*	13q12.11	20 826 877 – 20 994 706	ensured
RP11-523H24	13q12.11	19 098 345 – 19 306 540	ensured
RP11-1031I2	13q12.11	18 880 857 – 19 076 436	ensured
RP11-134H3	1312.11	18 758 361 – 18 901 819	ensured
RP11-933M24	13q12.11	18 413 567 – 18 600 943	ensured
RP11-825L14	9q13-9q21.11	70 367 430 – 70 586 283	ensured
RP11-151G22	9q13	70 224 828 – 70 389 343	ensured
RP11-2M11	9q13	70 027 036 – 70 186 337	ensured; plus several signals on chr9 and an additional on chr2

All BAC clones were found on the UCSC Genome Browser (http://genome.ucsc.edu/index.html) except those marked with an asterisk (*) which were chosen with the help of the BAC/PAC collection database of the University of Bari, Italy (http://www.biologia.uniba.it/rmc/).

A.5 mRNA sequence information

A.5.1 ETV6 transcript

GenBank Accession number NM_001987, Version NM_001987.4.

A.5.2 NTRK3 transcript

A.5.2.1 NTRK3 transcript variant 1

GenBank Accession number NM_001012338, version NM_001012338.1.

This isoform can be referred to as the full-length isoform (isoform A) and all other isoforms represent splice variants of it. One of the alternatively spliced transcripts (isoform B) is referred to in A.5.2.2.

A.5.2.2 NTRK3 transcript variant 3

GenBank Accession number NM_001007156, version NM_001007156.1.

Due to data base discrepancies this isoform appears as transcript variant 3 in the NCBI GenBank but is called isoform B in this work referring to the uniprot.org data base.

B. Abbreviations

AML	acute myeloid leukemia
BAC	bacterial artificial chromosome
bp	base pairs
CAM	chloramphenicol
cDNA	complementary deoxyribonucleic acid
(d)dATP	(di)deoxy adenosin triphosphate
(d)dCTP	(di)deoxy cytidin triphosphate
(d)dGTP	(di)deoxy guanosin triphosphate
(d)dNTP	(di)deoxy nucleotide triphosphate
(d)dTTP	(di)deoxy thymidin triphosphate
DEPC	diethyl pyrocarbonat
DNA	deoxyribonucleic acid
dT	deoxy thymidine
DTT	dithiothreitol
dUTP	deoxy uridin triphosphate
E.coli	Escherichia coli
FISH	fluorescence in-situ hybridization
FITC	fluorescein isothiocyanate
Kan	kanamycin
kb	kilo base pairs
LB	Luria Bertani
mb	mega base pairs
M-MLV	Moloney murine leucemia virus
MTX	methotrexate
OD_{260}	optical density at a wavelength of 260 nm
PCR	polymerase chain reaction
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
rpm	rotations per minute
RT	reverse transcriptase
U	units

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A Translocation between Chromosome 12 and Chromosome 15 leads to a novel ETV6-NTRK3 Fusion Transcript in Acute Myeloid Leukemia

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Introduction: Chromosomal translocations involving two or more chromosomes are found in many hematological malignancies. As a consequence fusion genes arise which may provide the cell with proliferation advantages. In this study the ETV6 (ETS variant gene 6) gene is involved in a translocation event in a 54-year-old AML patient and the aim was to identify the partner of the resulting fusion gene. **Methods:** After morphological diagnosis of AML cells of peripheral blood and bone marrow were analyzed for surface and cytoplasmatic marker expression by flow cytometry (Becton Dickinson, CA). Conventional karyotyping (GTG-banding) was performed using bone marrow cultures. In addition to monosomy 7 a translocation involving the ETV6 gene was proven by ETV6 break apart FISH. A 3´RACE-PCR was performed to characterize the unknown 3´end of the fusion product. The PCR products were subsequently cloned and sequenced. A reverse transcriptase PCR was used to verify the rearrangement. **Results:** In a 54-year-old male patient with recurrent infections and anemia AML with minimal differentiation (FAB M0) and multilineage dysplasia was diagnosed. Immunological phenotyping of the blasts confirmed a CD34 pos., CD13 pos., CD33 pos., CD56 pos., CD7 pos., CD2 pos., MPO negative population. Whole chromosome paints confirmed a t(12;15)(p13;q25). An ETV6 break apart FISH demonstrated an involvement of this gene. A 3´RACE-PCR and subsequent sequencing analysis identified NTRK3 (neurotrophic tyrosine receptor kinase 3) as in-frame fusion partner of the ETV6 gene. NTRK3 is located on chromosome 15q25.3 and its transcripts encode either catalytically active proteins or truncated isoforms that lack the intracellular kinase domain. The ETV6-NTRK3 transcript fuses ETV6 exons 1 through 5 with exons 13b and 14b of a truncated NTRK3 isoform and encodes a protein that contains the amino-terminal HLH domain of the ETV6 protein and C-terminal amino acids 529-612 of the NTRK3 protein.
Treatment of the patient with Alexan (100mg/m^2 d 1-7) and Idarubicin (12 mg/m^2 d 1-3) resulted in a partial remission. After HAM and FLAG-Ida and Anti-CD34 recombined monoclonal antibody (Myelotarg®) the patient died due to progression of the disease. **Conclusions:** We identified a novel ETV6-NTRK3 gene fusion in a patient with minimal differentiated AML. Interestingly, this chromosomal rearrangement is documented in a significant fraction of patients with secretory breast cancer and congenital fibrosarcoma.

Curriculum Vitae

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Johanna Kralik