

# BCR/ABL1 Extra Fusions in Patients with Chronic Myeloid Leukaemia (CML)

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## 1. Introduction

Chronic myelogenous leukemia (CML) is characterized by the Philadelphia chromosome (Ph), which is originated by a balanced translocation between chromosomes 9 and 22, that is,  $t(9;22)(q34;q11)$  results in production of a BCR-ABL1 fusion protein with constitutive Abl kinase activity. In experimental models, the expression of said activity causes expansion of pluripotent stem cells, preferentially favours myeloid lineage differentiation, and inhibits erythroid differentiation [1,2].

The BCR/ABL1 fusion in 22q11 is observed in ~95% of patients with chronic myeloid leukemia (CML). The BCR/ABL1 fusion signal on the derivative chromosome 22 is present in most CML patients.

In 5-10% of cases, this signal is generated by variant rearrangements (variant Ph) that involve other genomic regions [3-7]. More than 600 CML cases with variant rearrangements have been reported. The breakpoint distribution clearly exhibits a non-random pattern, with marked clustering to some chromosome bands, such as 1p36, 3p21, 5q13, 6p21, 9q22, 11q13, 12p13, 17p13, 17q21, 17q25, 19q13, 21q22, 22q12, and 22q13, which suggests that these regions may be particularly prone to breakage. In addition, some specific variants are also known to be more frequent, with the two translocations  $t(3;9;22)(p21;q34;q11)$  and  $t(17;22)(q25;q11)$  both having been reported in more than 10 cases [8].

The occurrence of additional chromosomal aberrations (ACA) in Ph-positive CML is strongly associated with disease progression and has been interpreted as a sign of clonal evolution as well as chromosomal instability. Mitelman et al. [9] identified +Ph, +8, and  $i(17q)$  as the most common secondary changes that occur in nearly 90% of the cases with additional abnormalities. These three abnormalities were proposed to follow the 'major route' of clonal evolution, whereas other changes, which evolved more rarely, were suggested to follow the 'minor route' [9]; the terms 'major' and 'minor' refer to frequencies of the aberrations but not to the pathogenetic impact. Thus the three major route changes, followed by +19, were the most common ACA. Other additional chromosomal abnormalities take place in less than 10% of the cases, the most frequent being -Y, +21, +17, -7, and -17. On the other hand, no apparent differences in ACA between CML with standard Ph and CML cases with Ph variants are observed. The most common additional chromosomal changes are +8 (34%), +Ph (30%),  $i(17q)$  (20%), +19 (13%), -Y (8% of

anomalies), +21 (7%), +17 (5%), and -7 (5%), when these two CML groups are combined (Table 1).

Chronic Phase (in order of appearance)	Accelerated and Blastic Phase (in order of appearance)
+Ph classical	+Ph classical
+Ph variant	+ Ph variant
+ Ph, +8	+Ph, +8
+Ph, -Y	+Ph, -Y
+Ph, -21	+Ph, i(17q)
+8	+19/ +17 /-7
Complex Karyotype	Complex karyotype

Table 1. Secondary chromosome abnormality patterns in CML patients.

The chromosome bands frequently involved in secondary structural changes are similar in both groups. It is apparent that some chromosome segments are particularly 'breakprone': 1q, 3q21, 3q26, 7p, 9p, 11q23, 12p13, 13q11-14, 17p11, 17q10, 21q22, and 22q10 when breakpoint distribution is observed in CML with standard Ph.

The ACA are found in various combinations and it has been suggested that i(17q) followed by +8 are rather early changes, whereas trisomy 19 is a relatively late one [10,11]. Although this temporal order of ACA seems common, exceptions clearly exist. There are several examples of Ph-positive CML cases with +8 in all metaphases but i(17q) may also occur in only subclones, and Ph-positive CML cases with a trisomy 19 [12]. Moreover, some combinations are more frequent than others. While +8, +Ph, and i(17q) often occur together, the combination i(17q),+19 (without +8/+Ph) has only been reported in 2 cases; +8,i(17q),+19 (without +Ph) in 16 cases; and i(17q),+19,+Ph (without +8) in 2 cases[12]. Some combinations (+8, i(17q); +8,+19; +19,+Ph) are positively associated, while i(17q),+19 and i(17q),+Ph are negatively associated according to Hashimoto et al. [13].

Thus, the selective ACA appearance seems to vary, depending on other aberrations present in the combination. Furthermore, available data indicate that the most common secondary abnormalities occur in a step-wise, well-ordered manner, with a putative frequent pathway which starts with i(17q), then followed by +8 and +Ph, and lastly +19. Since three out of the four most frequent ACA involve gains, i.e. +8, +19, and +Ph, thus hyperdiploidy is the most common modal number in CML with ACA [14,15]; nearly 50% of the cases have 47-50 chromosomes. Pseudodiploidy (46 chromosomes with structural abnormalities) is observed in almost 40% of the cases, while other modal numbers are uncommon. It is worth bearing in mind that the distributions of ploidy levels are not different between CML cases with standard Ph and those with variant Ph translocations, which confirms a strong indication that there are not any major differences between these two groups when related to cytogenetic evolution patterns. Most of the ACA in CML are genomically unbalanced, such as trisomies, monosomies, and deletions. In fact, this is common in hematologic malignancies, therefore while the primary changes have been proposed as balanced, the secondary changes are said to be unbalanced [16]. However, there are some notable exceptions in CML, in 10 cases the presence of t(15;17)(q22;q12-21) has been reported [12]. Other balanced rearrangements which are characteristically found in acute myeloid leukemia (AML) or myelodysplastic syndromes (MDS) and occasionally also found in CML

BC include:  $\text{inv}(3)(\text{q}21\text{q}26)/\text{t}(3;3)(\text{q}21;\text{q}26)$ ,  $\text{t}(3;21)(\text{q}26;\text{q}22)$ ,  $\text{t}(7;11)(\text{p}15;\text{p}15)$ ,  $\text{t}(8;21)(\text{q}22;\text{q}22)$ , and  $\text{inv}(16)(\text{p}13\text{q}22)$  [12, 18-22].

Although secondary by definition, these ACA do not behave as ordinary secondary changes. Firstly, they are strongly related to quite specific phenotypic features as the  $\text{i}(17\text{q})$  have been associated with marked basophilia and the presence of pseudo Pelger-Huët neutrophils [4], while other secondary aberrations are not. Secondly, they do not normally take place at the same time as other common additional changes, such as +8,  $\text{i}(17\text{q})$ , and +Ph, which clearly indicates that they follow a different evolutionary pathway. Rather than being secondary changes, they may be seen as second primary changes. It is worth noting that the fact that balanced aberrations characteristic of acute lymphoblastic leukemia (ALL), such as  $\text{t}(4;11)(\text{q}21;\text{q}23)$  and  $\text{t}(8;14)(\text{q}24;\text{q}32)$ , have, to date, never been reported in CML blastic phase [12]. The idea that ALL-associated translocations cannot originate during the particular differentiation stage that characterizes the Ph-positive stem cell population is a possibility. On the other hand, and equally possible, ALL-associated translocations may not provide any selective advantage for cells expressing the BCR/ABL1 chimeric protein.

In 60–80% of the cases, ACA precede or accompany blastic phase [14, 15, 23-33], thus there is undoubtedly a strong association between secondary changes and transformation. Furthermore, early studies of splenic CML transformations give rise to strong support for the clinical importance of additional changes, showing that the secondary aberrations appeared in the spleen before bone marrow [34-36]. However, the prognostic impact of ACA has turned out to be difficult to assess. First, although most studies have reported that lack of ACA during blastic phase give rise to a better prognosis [32, 33, 37-41], some investigators have not found any prognostic differences between CML blastic phase cases with and without secondary changes [23, 30]. Second, it has been debated whether additional changes found during chronic phase constitute a dismal prognostic sign, announcing transformation. On one hand, as has been repeatedly highlighted, the emergence of new clones does not necessarily lead to disease progression and some clones appear only transiently [23, 42, 43]. On the other hand, the appearance of new ACA or increment of the clone with additional abnormalities has been shown to be generally associated with clinical deterioration [28, 37, 44].

Moreover, Sokal et al. [45] reported that patients' survival with secondary changes already present at the time of diagnosis had a shorter survival rate, but with survival curves for those with and without additional changes not diverging until after the 2-year point. Third, the number and types of aberrations present during chronic phase and blastic phase may also play a role. Quite often the karyotypic changes observed in the chronic phase are single events, particularly the loss of the Y chromosome, +8, +Ph, and  $\text{i}(17\text{q})$  [47], while they are multiple and complex in blastic phase [15,31,37,42,46]. The aberrations in  $\text{i}(17\text{q})$ , or other changes that result in loss of 17p seem particularly ominous [15,23,25,37,42,47-49]. Furthermore, trisomy 8 and +Ph that take place in the blastic phase have been associated with poor prognosis [15, 50-52] (table 1). Thus, the prognostic impact of secondary abnormalities in CML is complex, heterogeneous, and is probably related to several parameters such as time of appearance and specific abnormalities [52].

Therefore, response to imatinib on the one hand, and resistance on the other, may also be conditioned by different types of ACAs. Enhanced response or resistance to treatment may be associated with the occurrence of different cytogenetic or molecular changes which are capable of activating the metabolic pathways with different outcomes [53,54]. Several

studies have assessed the impact of ACA on the clinical efficacy of imatinib by assuming that these abnormalities may confer BCR/ABL1-independent proliferation and decrease sensitivity to imatinib [50-55].

However, the presence of additional copies of the BCR/ABL1 fusion onto structurally abnormal chromosomes has rarely been reported in the chronic or blastic phase of CML patients.

We present two CML patients with additional copies of BCR/ABL1 fusions: the first patient with +Ph and BCR/ABL1 extra fusion in chromosome 18 in the blastic phase, and the second patient with four BCR/ABL1 fusion signals at diagnosis .

## 2. Case report n° 1

A 34-year-old patient was diagnosed with CML. The hematologic features were: Hb, 96 g; WBC,  $354 \times 10^9/L$  with 2% blasts, 4% basophils; platelets,  $716 \times 10^9/L$ . Neutrophil alkaline phosphatase (NAF) activity was absent. The bone marrow aspirate was hypercellular with 1% blast cells.

A cytogenetic study was performed on bone marrow cells and revealed a 46,XY,t(9;22)(q34;q11) karyotype in all cells analyzed. FISH was performed on chromosome preparations using commercially available probe sets (LSI BCR/ABL-ES probe; Vysis, Downers Grove, IL) and based on a count of 100 cells (metaphases and interphases). The existence of the classic BCR/ABL1 fusion was proved. The patient was treated with hydroxyurea and  $\alpha$ -interferon, with a satisfactory clinical, but unsatisfactory cytogenetic and FISH response , the maximum response reached, based on a count of 120 cells, was 27/120 cells with the BCR/ABL1 rearrangement during the 4 year follow-up . A bone marrow transplantation could not be performed because no compatible HLA-matched donor was available.

After the aforementioned, 4 years after the initial diagnosis, a decrease of Hb, hematocrit, and platelets took place in the peripheral blood: WBC was  $4.8 \times 10^9/L$  with 38% blasts. Bone marrow was hypercellular, with 30% of cells representing the erythroid lineage at all the maturation stages, and with intense dyserythropoietic changes: 21% in the myeloid series (myeloid/erythroid ratio 5:1.5); 9% in the lymphoplasmocytoid series; and 40% as large blasts with fine chromatin in the nucleus, with two nucleoli, and irregularly shaped nongranular cytoplasm. Blast cells gave positive histochemical reactions for peroxidase and naphthol AS-D chloracetate esterase (inhibited by fluoride) and were periodic acid Schiff (PAS) positive as granules. Analysis in bone marrow of more than 100 cells at this time revealed that 94% of the cells had two fused BCR/ABL signals: one on the Ph chromosome and the other on the short arm of a derivative chromosome 18, detected by metaphase FISH analysis with Vysis LSI-BCR/ABL-ES and Cytocell mBCR/ABL probes (Fig. 1). Karyotype in bone marrow samples showed one Ph chromosome and one normal chromosome 22, and one normal chromosome 18 and another with a deletion of part of its short arm. The remaining 6% of cells only had the usual BCR/ABL1 fusion, without any other abnormalities.

The patient was then treated with mitoxantrone and cytarabine without clinical response, and later with splenic radiotherapy, which led to an intense pancytopenia; the patient then died 4 years 8 months after the initial diagnosis.

The occurrence of additional chromosome changes is an ominous sign, indicating that illness progression is imminent; however, the etiology of blastic transformation is still not

completely clear. Erythroblastic transformation of CML has been reported in 10% of patients in blastic crisis; nonetheless, this has not been clearly associated to specific chromosome aberrations [3-7, 61-66].

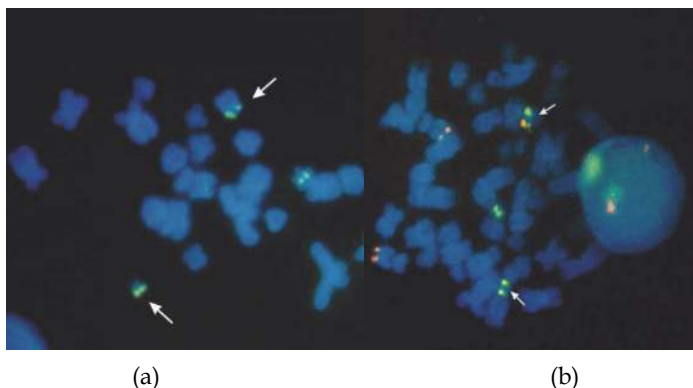


Fig. 1. (a) Metaphase cell FISH showing two fusions (a classic one in the Ph chromosome and another BCR/ABL fusion in 18p) (arrows), two orange signals (normal and residual ABL probe in 9q34), and one green signal (BCR in the normal 22 chromosome) (94% of cells). (b) Partial FISH metaphase with DAPI/ FITC showing the three copies of the BCR gene (in the Ph chromosome; in 18p, indicated by arrows; and in the normal chromosome 22) (94% of more than 100 cells).

Three cases have been described with additional BCR/ABL1 fusions onto 18p accompanied by other chromosome alterations [67, 68], two of which have been reported by the same authors [68]. In the case described by Tanaka et al. [67], the blast morphology was not specified. Khac et al. [68], however, described two patients with CML in blastic transformation with a duplication and transposition of BCR/ABL1 to 18p that presented loss of part of its short arm; both patients had erythroblastic transformations, which the authors believed to be associated with this particular cytological subtype of disease progression in CML. The present case supports the relationship suggested by Khac et al. [68] between a tendency for erythroblastic transformation and the presence of extra fused BCR/ABL1 onto 18p, as in the patient we described, with a duplication and transposition of BCR/ABL1 onto 18p in the majority line (95%) in blastic phase, with no other chromosome alterations, together with an increase of red cells, intense dyserythropoietic features and PAS in granules leading to a fatal outcome.

### 3. Case report n° 2

A 31-year-old caucasian man, a smoker (10 cigarettes/day) and with a past appendectomy, presented with a history of abdominal pain with diarrhea and an abscess in the jawbone; he was attended at the Department of Hematology. Clinical examination revealed hepatosplenomegaly. Complementary tests revealed leukocytosis in a peripheral blood examination. His white blood cell count was  $52 \times 10^9/L$ , with a peripheral monocytosis of  $18 \times 10^9/L$  and a left shift. The number of platelets was  $94 \times 10^9/L$ ; hemoglobin was normal; leukocyte alkaline phosphatase was reduced. In the biochemical

study (which included lipids and thyroid hormones) increased HDL, vitamin B12 and uric acid were observed; findings of the remaining studies were normal. The thoracoabdominal computed tomography scan revealed mild bilateral axillary adenopathy, right pleural effusion, hepatomegaly, and periaortic adenopathy; the gastroduodenal endoscopy was unremarkable. The patient's bone marrow was described as hypercellular, without visible fat spaces; megakaryocytes were present, normal in both number and morphology. The myeloid/erythroid ratio was 20:1. Myeloid precursors were high in number and left-shifted, with a relative increase in promyelocytes (7%) and eosinophils (6%). The blast percentage was 4%. Erythroid precursors were present in normal numbers.

Cytogenetic analysis with G-banding was performed on chromosomes obtained from 24-hour bone-marrow cell cultures following the standard procedures. Fluorescence in situ hybridization (FISH) was performed on chromosome preparations using a commercially available dual-color LSI BCR/ABL-ES probe (Abbott Molecular, Des Plaines, IL) labelled in SpectrumGreen and SpectrumOrange, respectively, to detect the BCR/ABL1 rearrangements in interphase and metaphase nuclei. To identify the chromosomes 9 correctly, a Vysis whole chromosome paint (WCP 9) was used, labeled in Spectrum- Orange. The hybridization was performed according to the manufacturer's protocols.

Molecular studies using real-time polymerase chain reaction (RT-PCR) from peripheral blood were performed. One microgram of the total RNA was used for cDNA synthesis, and later PCR was performed based on a Biomed protocol and using a LightCycler 2.0 [69] (Roche Diagnostics, Indianapolis, IN; Mannheim, Germany). A RT-PCR study with a fluorescence resonance energy transfer (FRET) probe quantification kit t(9;22) (Roche) that detected BCR/ABL1 fusion transcripts resulting from the major and minor breakpoint cluster regions (M-BCR and m-BCR) was also performed. The primers used in this kit were specifically designed to detect b3a2, b2a2, and e1a2 fusion transcripts, thereby covering >95% of the described t(9;22) translocations.

At diagnosis, cytogenetic studies indicated the following karyotype:

46,XY,der(22)t(9;22)(q34;q11.2)[3]/48,XY,+9,t(9;22)(q34;q11.2)x2,+22[40] (figure 2A).

FISH studies on 167 metaphase and interphase cells revealed the presence of a mosaicism formed by three distinct cellular clones at diagnosis. In the major clone, 150/167 cells (90%) presented four BCR/ABL1 fusion signals, with two signals on the two chromosomes der(9) and the other two signals on the two Ph chromosomes (Fig. 2B and 3). Three chromosomes 9 were observed with a WCP-9 probe. A sole BCR/ABL1 fusion signal, on chromosome 22, was observed in 5/167 cells (3%), and there were no rearrangements in the remaining cells analyzed (12/167, or 7%). The FISH technique was performed twice, confirming the results. The RT-PCR demonstrated a p210, b3a2 fusion transcript.

Once the patient was diagnosed as CML, treatment with imatinib was initiated at a dose of 400 mg/day. Two months later the patient was asymptomatic and in hematological remission. The b3a2 transcripts detected by RT-PCR were at 59%. The patient continued with the same imatinib treatment of 400 mg/day. Ten months after beginning therapy, a FISH follow-up study performed on a bone marrow sample revealed a cellular mosaicism, with partial remission. The initial majority line with four fusions was present in only 20/300 cells, only one BCR/ABL1 rearrangement was seen, on chromosome 22, in 25/300 cells; the remaining 255/300 cells were normal, without any fusion signals. The

p210 BCR-ABL1 fusion transcript, identified using RT-PCR (Biomed protocol), decreased to 1.66%.

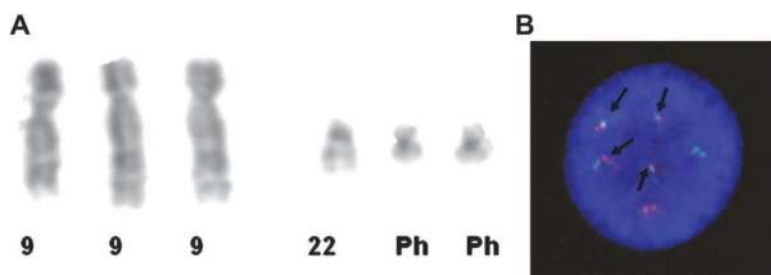


Fig. 2. (A) Partial metaphase karyogram shows two derivative chromosomes 9 and two derivative chromosomes 22, along with one each of normal chromosomes 9 and 22 in case report n° 2 of chronic myeloid leukemia in chronic phase. (B) For the same patient, interphase FISH with LSI BCR/ABL-ES probes (Vysis; Abbott Molecular, Des Plaines, IL) shows four BCR/ABL1 fusion genes (arrows). Filter: 4', 6-diamidino-2-phenylindole-rhodamine-fluoresceinisothiocyanate

A year after diagnosis, in a routine evaluation, the patient was still asymptomatic and in hematological remission. The RT-PCR study performed using FRET probes detected BCR/ABL1 transcripts. A quantification analysis (Biomed protocol) performed then was negative for p210 (b3a2), but p190 (e1a2) was found at 23.3%. Thus, the original sample was analyzed again, in which p190 was also positive. The original imatinib treatment was changed to dasatinib at 100 mg/day due to the disappearance of p210 and the presence of p190 in 23.3%.

At diagnosis, the patient in the present case had a karyotype with 48 chromosomes and four BCR/ABL1 fusion signals distributed between the two Ph chromosomes and two out of the three chromosomes 9 in 90% of all the cells analyzed by FISH. One possible explanation could be that the classical BCR/ABL1 fusion occurs initially, followed first by the duplication and translocation of the fusion to a derivative 9 chromosome and then by a duplication of both Ph chromosomes and 9q+ (the remaining chromosomes were unaltered). The possible poor prognostic effect of a BCR/ABL1 fusion located on chromosome 9 has been described [4, 5, 61, 70]. Nevertheless, other researchers have reported cases of patients with a chimerical BCR/ABL1 fusion gene on chromosome 9 in ~80% of CML samples [62] and a double fusion on both chromosomes 9 [71, 72], and the clinical course of the disease seemed unaffected by the positional regulation. In the present case, there was only hematologic remission after treatment. With regard to treatment with imatinib, additional copies of the Ph chromosome have been reported [73, 74]. The development of additional chromosomal aberrations (ACA) in Ph negative or Ph+ cells during treatment is a sign of clonal evolution and of treatment failure. Patients with ACA more frequently tended to have BCR/ABL1 mutations (53% versus 29%) and more resistant mutations (60% versus 38%) [75]. The acquisition of ACAs may confer resistance to imatinib mesylate a mechanism for imatinib resistance. Therefore, response to imatinib on one hand and resistance on the other may also be conditioned by different types of ACAs. Enhanced response or resistance to treatment may be associated with the occurrence of different cytogenetic or molecular changes that are able to activate metabolic pathways with different outcomes [76-77].

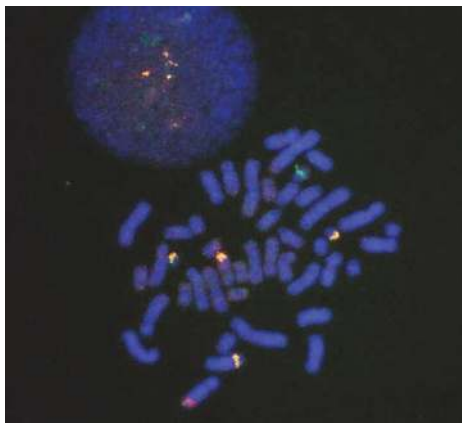


Fig. 3. Metaphase FISH with Vysis LSI BCR/ABLeES probes shows four BCR/ABL fusion signals (yellow): two signals on the two der(9) chromosomes and two signals on the two der(22) chromosomes. The green and orange signals indicate, respectively, the BCR gene on the normal chromosome 22 and the ABL gene on the normal chromosome 9.

In the present case, all the cytogenetic aberrations began to decrease with imatinib treatment, and only a small percentage of cells maintained the initial karyotype.

At the molecular level, the patient showed a p210 and p190 fusion. A p210 decrease coincided with an important partial cytogenetical remission of the four BCR/ABL1 fusions. During evolution of the disease, the p210 protein disappeared with imatinib as first-line treatment, but p190 increased until treatment with dasatinib was started.

Five months after the start of treatment with dasatinib, RT-PCR showed that p210 remained negative and p190 had decreased to 1%. Two years after diagnosis, the patient was still clinically asymptomatic and hematologically in remission. New FISH studies, with 200 cells analyzed, also showed complete cytogenetic remission. Molecular studies remained negative for p210 and positive for p190 in only 0.09%.

Although interphase FISH is increasingly used for BCR/ABL1 gene rearrangement identification in CML, we believe, in agreement with Lim et al. [72] and Primo et al. [79] and supported by the present case, that the exact interpretation of any atypical interphase FISH pattern is dependent on FISH metaphase studies and molecular breakpoint definition of BCR/ABL1 fusion.

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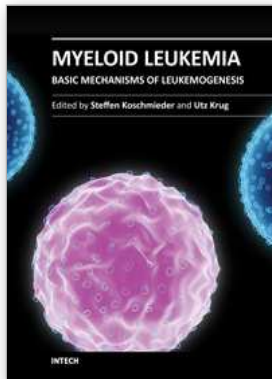
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## **Myeloid Leukemia - Basic Mechanisms of Leukemogenesis**

Edited by Dr Steffen Koschmieder

ISBN 978-953-307-789-5

Hard cover, 484 pages

**Publisher** InTech

**Published online** 14, December, 2011

**Published in print edition** December, 2011

The current book comprises a series of chapters from experts in the field of myeloid cell biology and myeloid leukemia pathogenesis. It is meant to provide reviews about current knowledge in the area of basic science of acute (AML) and chronic myeloid leukemia (CML) as well as original publications covering specific aspects of these important diseases. Covering the specifics of leukemia biology and pathogenesis by authors from different parts of the World, including America, Europe, Africa, and Asia, this book provides a colorful view on research activities in this field around the globe.

### **How to reference**

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Maria Teresa Vargas (2011). BCR/ABL1 Extra Fusions in Patients with Chronic Myeloid Leukaemia (CML), Myeloid Leukemia - Basic Mechanisms of Leukemogenesis, Dr Steffen Koschmieder (Ed.), ISBN: 978-953-307-789-5, InTech, Available from: <http://www.intechopen.com/books/myeloid-leukemia-basic-mechanisms-of-leukemogenesis/bcr-abl1-extra-fusions-in-patients-with-chronic-myeloid-leukaemia-cml>

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