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Oncogenes and cancer monitoring

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Abstract - Activation of cellular oncogenes by chromosomal translocation plays an important role in the development of several leukemias and lymphomas. One of the best studied examples is activation of the *c-abl* oncogene by the Philadelphia (Ph) translocation, t(9;22)(q34;q11), in Chronic Myelogenous Leukemia (CML).
In this paper the Ph translocation in CML is used as a model system to demonstrate the role oncogenes can play in human cancer. We will discuss how specific markers : The Ph chromosome, *bcr-abl* gene, *bcr-abl* mRNA, and bcr-abl protein (p210), can be used for diagnosis and monitoring of the disease during chemotherapy, interferon treatment or after bone marrow transplantation.

ONCOGENES AND THEIR ROLE IN LEUKEMIA

About twenty years ago analysis of transforming RNA tumor virusses led to the identification of unique transforming genes, called viral oncogenes, within the retroviral genome (ref. 1). These viral oncogenes appeared to be derived from homologous cellular genes present in normal DNA of the host cell. These sequences have been incorporated into the retroviral genome during transit of the retrovirus through the host cell. The eukaryotic homologs have been termed proto-oncogenes or cellular oncogenes (ref. 2). They play a role in regulation of growth and differentiation of normal cells.
However, when these proto-oncogenes are altered by mutation, translocation or amplification, their normal function is disturbed, leading to transition of proto-oncogenes into their oncogenic counterparts (oncogenes).
Further evidence for an important role of oncogenes in cancer was provided by 1) transfection studies (ref. 3, 4), 2) the fact that viral oncogenes cause cancer in animals and 3) mapping studies showing the presence of oncogenes near translocation breakpoints on chromosomes in cancer cells (ref. 5).
The best studied example of oncogene activation by chromosomal translocation in cancer is the activation of the *c-abl* gene by the Ph translocation in CML.

CLINICAL FEATURES OF CHRONIC MYELOGENOUS LEUKEMIA (CML)

CML is a hematopoietic malignancy arising from neoplastic transformation of a pluripotent bone marrow stem cell.
At diagnosis standard findings are leucocytosis, increased granulopoiesis, the presence of immature progenitor cells in peripheral blood, basophilia, and hepatosplenomegaly.
The course of the disease is biphasic. The initial chronic phase lasts for 1-4 years, is characterized by leukocytosis with full maturation, and can be controlled by chemotherapy. Invariably a blast crisis follows, in which differentiation is blocked and usually the cells are therapy resistant.

CYTOGENETICS OF CML

In 1960 Nowell and Hungerford demonstrated the presence of a minute chromosome, named Philadelphia (Ph) chromosome, in patients with CML (ref. 6). In 1973 Rowley identified the origin of the Ph chromosome i.e. the reciprocal translocation t(9;22) (q34;q11) (ref. 7). The Ph translocation is present in more than 90% of the CML patients (ref. 8).

In approximately 5% of the CML cases a variant form of the Ph translocation is found, involving chromosome 9, 22, and one or more other chromosomes (ref. 9, 10).
In CML blast crisis often additional chromosomal abnormalities are detected even before clinical deterioration becomes apparent. The most frequent additional chromosomal abnormalities are +8, i(17q), and +22q- (ref. 11). In 1987 a new translocation, t(3;21), has been identified as additional chromosomal aberration in CML accelerated phase and blast crisis (ref. 12).
In less than 5% of all CML patients no Ph chromosome is detected. These patients are called Ph-negative. Some of these Ph-negative CML patients have a less favourable prognosis as compared to Ph-positive CML (ref. 9, 13-15).

MOLECULAR BIOLOGY OF CML

The molecular rearrangement underlying the Ph translocation was discovered to be the translocation of the c-abl gene from chromosome 9 band q34 to the Ph chromosome (Fig. 1) (ref. 16).

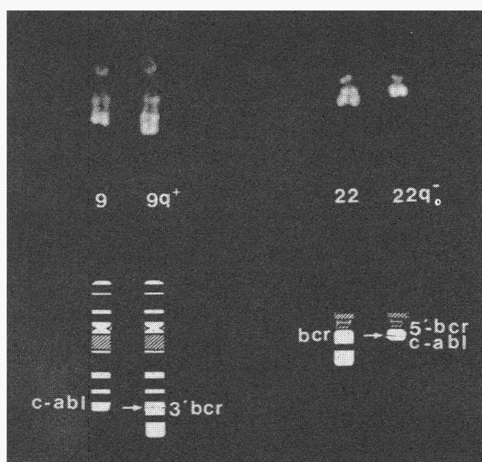

Fig. 1.
Top: Partial karyotype of standard Ph translocation t(9;22)(q34;q11) using R banding technique.

Bottom: Scheme of molecular alterations caused by t(9;22) in CML. The regional localization of 5'-bcr, 3'-bcr and c-abl genes are indicated.

On chromosome 22 the breakpoint is located in band q11 in the bcr gene.
In 1984 Groffen et al reported that in CML all breakpoints in the bcr gene were clustered in a region of 5.8 kb, the breakpoint cluster region (BCR) (ref. 16). The BCR region harbors three exons of the bcr gene (b1-b3), and nearly all breakpoints are located either between exons b2 and b3 or between exons b3 and b4.
In the abl gene breakpoints are scattered over a stretch of 200 kb (ref. 17).
As a result of the Ph translocation a new chimeric bcr-abl gene on the Ph chromosome is generated (ref. 18), which is transcribed into a chimeric bcr-abl mRNA (ref. 19, 20), encoding a 210 kD bcr-abl protein (p210) (ref. 20, 21). This p210 has enhanced tyrosine kinase activity and is thought to play a key role in leukemogenesis (ref. 22-24).
Depending on the localisation of the breakpoint in the bcr gene, BCR exon b3 sequences can either be absent or present in the chimeric bcr-abl mRNA. In its absence BCR exon b2 is joined to abl exon a2 (termed b2a2 mRNA). Otherwise BCR exon b3 is joined to abl exon a2 (termed b3a2 mRNA).

Each of these mRNAs is translated into protein. The two corresponding 210 kD bcr-abl proteins (ref. 22-24, 25) will differ in 25 amino acids encoded by exon b3.

Interestingly the disruption of the abl protein, leading to the activation of its tyrosine kinase activity in CML is comparable with the formation of the gag-abl fusion protein found in Abelson Murine Leukemia virus. This virus causes lymphoma in mice (ref. 26) and its activity is directly dependent on the presence of the v-abl protein in which the N terminal part of the abl polypeptide is substituted by a gag viral moiety, resulting in a gag-abl fusion protein with enhanced tyrosine kinase activity and tumorigenicity (reviewed in ref. 27, 28, 29).

An obvious question is: Does p210 cause CML?
Several attempts to express p210 in primary bone marrow cultures have yielded only lymphoid transformants in vitro, even under conditions favouring myeloid cell proliferation (ref. 30, 31). Transgenic mice carrying a bcr-v abl fusion gene driven by an immunoglobulin enhancer or retroviral promotor developed lymphoid malignancies only (ref. 32). Final proof that bcr-abl protein induces CML came from experiments by Daley et al (ref. 33). They transplanted irradiated mice with bone marrow that has been infected with a retrovirus encoding p210. The recipient mice developed a myeloproliferative syndrome closely resembling CML.

TECHNIQUES USED TO DETECT TUMOR SPECIFIC MARKERS IN CML

The cytogenetic and molecular characteristics of CML show that in nearly all CML patients the leukemic cells are characterised by the following markers: the Ph chromosome, bcr-abl mRNA, and p210 bcr-abl protein. These markers can be used for CML diagnosis and monitoring of the disease during chemotherapy, interferon treatment or after bone marrow transplantation. For this purpose the following techniques are used.

Chromosome analysis is performed to detect the Ph, 9q+ chromosome and additional chromosomal aberrations. Following established procedures blood and bone marrow cells are cultured for 24 and 48 hours without stimulation (ref. 34), harvested, stained and analysed.

The Southern blot technique is applied to demonstrate the breakpoint in the BCR region of the bcr gene. DNA is extracted from blood and bone marrow cells, digested with restriction enzymes, electrophoresed through an agarose gel, and blotted onto nylon membrane, followed by hybridisation to ^{32}P labelled BCR specific probes. Autoradiograms are made (ref. 16, 35). Detection of aberrant sized restriction fragments mark the presence of a breakpoint in the BCR.

Pulsed field gel electrophoresis (PFGE) is performed when the breakpoints are expected to be scattered over a large region e.g. as is the case in the abl gene: blood or bone marrow cells are mixed with low melting point agarose, poured in a mould in order to form blocks. Thereafter cells in the blocks are lysed, treated with proteinase K, and high molecular weight unsheared DNA is made. After digestion with rare cutting restriction enzymes, the blocks are placed in the slots of a 1% agarose gel, and pulsed field gel electroforesis is carried out. After blotting onto nylon membrane and hybridisation to specific probes autoradiograms are made. Detection of aberrantly sized restriction fragments mark the presence of a breakpoint in the gene investigated. Using this technique fragments variing in size from 4-1200 kb can be detected (ref. 36).

In situ hybridization is used to demonstrate the chromosomal localisation of the gene of interest. The same chromosomal slides are used as for chromosomal analysis. After denaturation, hybridisation to radioactive or non radioactive labelled probe and stringent washing, the chromosomal localisation of the probe is determined (ref. 37).

The Polymerase Chain Reaction (PCR) is performed to detect bcr-abl mRNA.
Total RNA is extracted from blood or bone marrow cells (ref. 38).
cDNA preparation and amplification with the PCR are performed as described by Hermans et al (ref. 39). The primers used in the PCR reaction are schematically drawn in Fig. 2. In short: using a sense primer containing b1b2 sequences (primer2) and an antisense primer containing a3 sequences (primer 1) a fragment is amplified covering the bcr-abl junction region. As an internal positive control, half of the cDNA product is used to amplify an abl fragment, that should always be present irrespective of the Ph translocation. To amplify this abl fragment a sense primer containing a2 sequences (primer 3) and an antisense

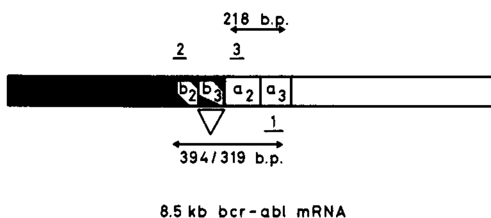

Fig. 2.
Scheme of the 8.5 kb bcr-abl mRNA showing the localization of bcr and abl primers.Using primer 1 and 2 a fragment of 394 or 319 bp is amplified, depending on the absence or presence of exon b3 in the mRNA (indicated by).
Primer 1 and 3 are used to amplify an abl fragment of 218 bp that should always be present irrespective of the presence of the Ph translocation.

primer containing a3 sequences (primer 1) are used. The amplified fragments are electrophoresed through a 2% agarose gel, blotted onto nylon membrane and hybridised to two ^{32}P labelled oligonucleotides, that will specifically recognize the bcr-abl fusion region b2a2 or b3a2 in the amplified fragment.

With the PCR technique as few as 1 Ph positive cell in 100,000 normal cells can be detected.

Autophosphorylation assays are used to demonstrate the presence of the bcr-abl protein. This protein, p210, is identified using specific antibodies that recognize the bcr-abl fusion epitope on the p210 molecule (ref. 40, 41).

APPLICATIONS

The presence of consistent chromosomal and molecular aberrations in CML provides the opportunity to screen patients at diagnosis for the presence of these abnormalities and to monitor the disease during treatment.
In our lab cytogenetic analysis is performed at diagnosis in all patients, who on clinical grounds are suspected to have CML. This analysis is repeated at acceleration or at blast crisis. In blast crisis often additional chromosomal abnormalities are detected before clinical deterioration takes place.
In 90% of the CML cases t(9;22) will be found. In these cases molecular investigations are not routinely performed.

In patients with variant Ph translocations, in which involvement of chromosome 9 and 22 is cytogenetically visible, no further molecular investigations are required. However, when involvement of one of these two chromosomes is not apparent under the microscope, either Southern blot analysis, in situ hybridisation or PCR is necessary to prove that bcr-abl rearrangement has taken place.

In Ph-positive CML patients whose Ph chromosome disappears during treatment, as is seen in patients treated with interferon, combination chemotherapy or after bone marrow transplantation, molecular investigations are used to detect minimal residual disease (ref. 42-45). Because Southern blot analysis has approximately the same sensitivity as chromosomal analysis, i.e. 5-10 % of the cells need to be Ph positive, PCR analysis (ref. 42-45), or in situ hybridisation using nonradioactively labelled BCR or abl probes (ref. 46, 47) are first choice in detection of minimal residual disease.

In Ph-negative CML patients Southern blot analysis and / or PCR analysis are always performed to identify patients who have the same bcr-abl rearrangement as Ph-positive CML. Investigations by our own group showed that using a combination of Southern blotting, Pulsed Field Gel Electrophoresis, and PCR bcr-abl rearangement could be demonstrated in 5 out of 12 Ph-negative CML patients (unpublished results and ref. 48, 49). Additionally, four of these patients were also studied using in situ hybridisation, showing complex Ph translocations in all four patients (ref. 48). Concurrent mapping of 5'BCR and c-abl in all four patients was in agreement with the presence of bcr-abl mRNA as detected by PCR analysis. In all of them three chromosomes were involved, indicating that complex Ph translocations had led to an apparently Ph negative karyotype. The presence of a bcr-abl fusion gene and expression of bcr-abl mRNA both in Ph-positive and in some cases of Ph-negative CML explains why clinically these patients have the same disease.

A different situation occurs in Ph-negative CML patients in which no bcr-abl rearrangement is found (ref. 49, 50). In this group another mechanism might be responsable for the disease e.g. Ras activation (ref. 51, 52). The latter cases have the least favourable prognosis and probably don't represent CML, but rather belong to another group of myeloproliferative diseases more resembling Chronic Myelomonocytic Leukemia (CMML).

The Ph chromosome is also found in acute leukemia, particularly in adult patients with Acute Lymphoblastic Leukemia (ALL) (ref. 53, 54). In some of these patients the same bcr-abl gene, mRNA, and protein are formed as in CML, but in others the breakpoint in the bcr gene is located more 5' i.e. in the first intron of the bcr gene, resulting in a different bcr-abl fusion gene (ref. 55). The latter is transcribed in bcr-abl mRNA in which bcr exon 1, termed e1, is fused to abl exon a2. This e1a2 mRNA encodes a 190 kD bcr-abl protein, p190, that also exhibits enhanced tyrosine kinase activity (ref. 56-58).

For diagnosis and detection of minimal residual disease in the Ph-positive acute leukemias the same strategy is followed as is described for CML. The only difference is, that additional probes and primers are used for in situ hybridisation, PFGE and PCR, that are specific for a breakpoint in the first intron of the bcr gene. The localisatión of the breakpoint in abl is the same as in CML. The p190 bcr-abl protein can be detected by autophosphorylation assays using anti BCR or anti abl antibodies or antibodies that specifically recognize the bcr-abl fusion epitope on the p190 molecule (ref. 59).

CONCLUSION

In CML the Ph chromosome, breakpoint in the BCR region, expression of bcr-abl mRNA and p210 protein represent unique markers specific for the leukemic cells. Nowadays these markers are succesfully used for diagnosis, follow up, and detection of minimal residual disease.

For many cancers and leukemias the molecular mechanisms are not yet unravelled, but recent results are hopeful. This year the breakpoints of t(1;19) in B-ALL (ref. 60, 61), t(6;9) in AML (ref. 62), and t(15;17) in APL (ref. 63, 64) have been cloned, providing new tools for diagnosis and monitoring of disease.

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