Expression of genes encoding centrosomal proteins and the humoral response against these proteins in chronic myeloid leukemia

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Abstract. As the extent of centrosome abnormalities in chronic myeloid leukemia (CML) correlates with disease stage and karyotype alterations, abnormal expression of genes encoding centrosomal proteins may be an early prognostic marker of disease progression. In the present study, we showed that in comparison with healthy controls, the expression of four centrosomal genes (AURKA, HMMR, PLK1 and ESPL1) in the peripheral blood of CML patients was significantly enhanced at diagnosis and decreased to the basal level in most patients treated with imatinib mesylate for three months. In the remaining patients (17%), this decrease was delayed and was associated with worse overall survival. The detection of antibodies in sera showed that patients with higher overall antibody production had superior outcomes in terms of achieving major molecular response and failure-free survival. These data suggest that the dynamics of the response of centrosomal genes should be considered as a risk factor and immunity against centrosomal proteins may contribute to treatment response.

Introduction

Chronic myeloid leukemia (CML) is a clonal disease of hematopoietic stem cells characterized by the Philadelphia chromosome (Ph) generated by the reciprocal translocation t(9;22)(q34;q11) resulting in the BCR-ABL1 fusion gene. A constitutively upregulated tyrosine kinase activity of BCR-ABL1 contributes to malignant transformation of cells (1). The disease is usually recognized in a chronic phase (CP) that may continue for several years. However, the progression of CP may lead to an advanced stage that includes an accelerated phase (AP) and blast crisis (BC). In most cases, CP is suppressed with imatinib mesylate (IM), a tyrosine kinase inhibitor (TKI) of the BCR-ABL1 protein. The resistance to IM that develops in a portion of patients in CP is mostly caused by a mutation in the kinase domain abolishing binding of IM to BCR-ABL1 or by amplification of the BCR-ABL1 gene (2).

Current molecular diagnostic of CML and evaluation of the disease response to therapy are based on the detection of the BCR-ABL1 transcript, monitoring of its level, and mutation analysis of the BCR-ABL1 kinase domain. Increase in the BCR-ABL1 mRNA level in CP is a marker of resistance to therapy, which suggests the risk of a relapse and the necessity of treatment modification (3,4). However, reliable markers of CML progression to predict disease prognosis and thus to modify the treatment protocol accordingly are still needed.

During CML progression, genomic instability in leukemic cells leads to the accumulation of mutations including chromosomal abnormalities, which are found in ~80% of CML patients (5). These genetic changes result in the generation of cells independent of the transformation potential of the BCR-ABL1 protein. Moreover, in BC, the second type of leukemia stem cells has been identified, granulocyte-macrophage progenitors responsible for rapid expansion of immature blast cells (6).

Centrosomes are cellular organelles that play an essential role in the separation of chromosomes into daughter cells. The development of malignant diseases is associated with centrosome aberrations that lead to genetic instability and chromosomal aberrations. In CML, the extent of centrosome abnormalities is correlated with disease stage and karyotype alterations (7).

Increased production of transcripts originating from various genes encoding centrosomal proteins has been confirmed as a negative prognostic marker in a variety of malignant diseases, but, with the exception of the hyaluronan-mediated motility receptor (HMMR) gene, their expression has not been tested in CML. Using RT-PCR, Schmitt et al (8) found HMMR mRNA expression in 75% of CML patients in...
CP and in 100% of advanced CML patients. They also found a correlation of HMMR and BCR-ABL1 expression in a patient with remission followed by relapse and demonstrated an HMMR-specific T cell response in 80% of patients. HMMR has been identified as a tumor-associated antigen (TAA) in acute myeloid leukemia (AML) and CML (9). Induction of HMMR-specific T cell responses has been found in patients with chronic lymphocytic leukemia (CLL) after immunization with a peptide vaccine (10).

Aurora kinase A (AURKA) that regulates centrosome duplication and separation is another centrosomal protein overexpressed in CML and a candidate vaccine target (11). As the AURKA gene is overexpressed or amplified in a variety of human tumors and kinase activity of the produced protein is involved in tumorigenesis, inhibitors of this kinase have been developed and tested in clinical trials (12).

Polo-like kinase 1 (PLK1) is implicated in numerous mitotic events including centrosome maturation. Similar to AURKA, it also has oncogenic potential and inhibitors of its kinase activity have been developed (13). In CML, the inhibition of PLK1 activity suppressed the proliferation of both IM-sensitive and IM-resistant leukemic cells (14).

The endopeptidase separase, the product of the extra spindle poles-like 1 (ESPL1) gene, plays a vital role in the separation of chromatids in mitosis. Its overexpression induced aneuploidy and tumorigenesis (15). Increased ESPL1 transcription in solid tumors is correlated with a high incidence of relapse and metastasis and with a lower survival rate (16). Moreover, a meta-selection of genomic datasets obtained from solid tumors identified ESPL1 as one of the four genes with the highest prognostic impact on survival outcome (17). In CML cells treated with IM, the separase level was reduced but its proteolytic activity was increased (18).

Overexpression of TAAs can be accompanied by serologic responses that show evidence of TAA immunogenicity. Simultaneously, if immunity contributes to cancer elimination, the generation of anti-TAA antibodies can be a positive prognostic marker. For instance, in a study of WT1 in myelodysplastic syndrome, an increased level of mRNA predicted worse survival, while a high level of antibodies was associated with longer survival (19).

In the present study, we measured the expression of four genes (AURKA, ESPL1, HMMR and PLK1) encoding centrosomal proteins and determined the production of antibodies against these proteins in CML patients treated with IM. We obtained data at diagnosis and during treatment and evaluated their significance for disease prognosis.

Materials and methods

Patients and samples. Patients diagnosed with CML and treated at the Institute of Hematology and Blood Transfusion, Prague, between 2010 and 2015 were included in the present study for prospective follow-up. In total, 36 patients newly diagnosed with CML in CP were enrolled. All but 2 patients were treated with 400 mg IM/day for at least 12 months. In one patient, this dose was reduced to 300 mg for two months due to side-effects and in one patient, IM was applied in a 600 mg dose from the seventh month of therapy. Thirty-three patients were followed up for at least 24 months and the remaining 3 patients for only 9, 12 and 13 months due to their death. Altogether, 5 patients died during the study, 2 of them due to progression of CML. Peripheral blood samples were taken at diagnosis and regular trimonthly monitoring for BCR-ABL1 was conducted during IM therapy. Blood samples of age- and gender-matched healthy controls were also collected. The characteristics of the study cohort are summarized in Table I. The response to IM treatment (optimal, warning or failure) was evaluated according to the European LeukemiaNet (ELN) recommendations based on the BCR-ABL1 transcript levels (20). The present study was approved by the Institutional Ethics Committee of the Institute of Hematology and Blood Transfusion, Prague and conducted according to the Declaration of Helsinki. All subjects signed the informed consent to participate in the present study.

Gene expression analysis. Total RNA was prepared from peripheral blood leukocytes using TRizol reagent, and cDNA
was synthesized by M-MLV reverse transcriptase (Promega, Madison, WI, USA) using random hexamer primers (21). The level of mRNA expression of BCR-ABL1 and genes encoding centrosomal proteins (called centrosomal genes in the present study) were analyzed trimonthly since the start of IM treatment by reverse-transcriptase quantitative real-time polymerase chain reaction (RT-qPCR). The β-glucuronidase gene (GUSB) that is used for standardized BCR-ABL1 monitoring in international scale (IS) units served as a reference gene also for expression analysis of centrosomal genes. BCR-ABL1 quantification was standardized within the European Treatment and Outcome Study (EUTOS) for the CML project of ELN and data were reported in the IS units. Primers and probes for BCR-ABL1 and GUSB were applied according to the Europe Against Cancer recommendations, and commercial plasmid standards were used to obtain calibration curves (Ipsogen, Ipswich, MA, USA). The transcripts of centrosomal genes were amplified in the following TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA): Hs01582073_m1 for AURKA, Hs00234864_m1 for HMMR, Hs00901772_m1 for ESPL1 and Hs00153444_m1 for PLK1. The qPCR was performed on Rotor-Gene 3000A (Qiagen, Hilden, Germany). Briefly, 10 µl of the PCR mixture contained 5 µl of TaqMan Universal Master Mix II, without UNG (Applied Biosystems), 0.5 µl of TaqMan Gene Expression Assay, and 2 µl of 3-fold diluted cDNA. After initial incubation at 50˚C for 2 min and polymerase activation at 95˚C for 10 min, each of the 40 cycles consisted of denaturation at 94˚C for 15 sec, and primer annealing and elongation at 60˚C for 60 sec. After amplification, analyses were performed using the Rotor-Gene software. The relative quantification of mRNA expression was evaluated by the ΔΔCq method (22). The mean gene expression in the healthy controls was used as a calibrator.

Recombinant proteins. Antigens for the detection of antibodies in an enzyme-linked immunosorbent assay (ELISA) were produced using a glutathione S-transferase (GST) gene fusion system in E. coli. Due to the size limitation of this system, partial sequences of the human genes AURKA (accession no. BC001280; nucleotides 600-1212 of the coding sequence), HMMR (BC108904; 1551-2178), PLK1 (BC014846; 4-670), and ESPL1 (NM_012291; 4-2106) were amplified from plasmids obtained from the Harvard Medical School (Boston, MA, USA) by Phusion High-Fidelity DNA polymerase (Finnzymes, Espoo, Finland) using the following primers containing XhoI restriction sites (underlined sequences): AURKA, 5′-TTAGCTCGAGGCTCATTAGTCTACCCAGAG TCTAC-3′ (forward) and 5′-TTAGCTCGAGGACTGTTTTG GCTAGCGTATCTTTTG-3′ (reverse); PLK1, 5′-TGTAGCTCGAGGCTCATTAGTCTACCCAGAG GCTAGCGTATCTTTTG-3′ (forward) and 5′-TTAGCTCGAGGCTCATTAGTCTACCCAGAG GCTAGCGTATCTTTTG-3′ (reverse); ESPL1, 5′-TGTAGCTCGAGGCTCATTAGTCTACCCAGAG GCTAGCGTATCTTTTG-3′ (forward) and 5′-TTAGCTCGAGGCTCATTAGTCTACCCAGAG GCTAGCGTATCTTTTG-3′ (reverse).

After digestion with XhoI (New England Biolabs, Ipswich, MA, USA), the PCR products were cloned into the pGEX-5X-1 vector carrying the GST gene (GE Healthcare, Little Chalfont, UK) and the cloned sequences were verified using the BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems). For the production of recombinant GST-fusion proteins, E. coli BL21-CodonPlus (DE3)-RIPL competent cells (Agilent Technologies, Santa Clara, CA, USA) were transformed with the constructed plasmids and incubated at 37˚C in Luria-Bertani medium (Duchefa, Haarlem, The Netherlands) with 100 µg/ml ampicillin and 2% glucose (Sigma-Aldrich, St. Louis, MO, USA). At an OD600 of 1.2-1.6, the production of recombinant proteins was induced by adding 0.3 mM isopropyl-β-D-thio-galactoside (IPTG; Duchefa) and the cells were incubated at 30˚C for 6 h. The bacterial pellets were resuspended in lysis buffer: 20 mM Tris-HCl (pH 8.0), 200 mM NaCl, 2 mM DTT, 200 mM protease inhibitor phenylmethylsulfonyl fluoride (PMSF; Serva, Heidelberg, Germany), and 0.5% N-lauroylsarcosine (Sigma-Aldrich) in the case of soluble GST-fusion proteins, HMMR, PLK1 and ESPL1. Since the GST-fusion protein AURKA was insoluble in this lysis buffer it was dissolved in a buffer with 2% N-lauroylsarcosine [10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 7 mM DTT, 200 mM PMSF and 2% N-lauroylsarcosine] (23). The resuspended cells were sonicated three times for 10-15 sec on ice and then lysed three times in the EmulsiFlex-C5 high pressure homogenizer (Avestin, Ottawa, Canada). The lysates were centrifuged (4˚C, 30 min, 16,000 x g) and the supernatants were mixed 1:1 with glycerol (Sigma-Aldrich) and stored at -20˚C. The production of recombinant proteins was verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

Detection of antibodies. Polysorb 96-well plastic plates (Nunc, Roskilde, Denmark) were coated overnight at 4˚C with glutathione casein (200 ng/well) in 50 mM carbonate buffer (pH 9.6) as previously described (24). Human sera were diluted 1:50 in blocking buffer [0.2% casein (Sigma-Aldrich) and 0.05% Tween-20 (Serva) in phosphate-buffered saline (PBS)] and incubated with the lysate containing the fusion antigen (Ag) or only the GST tag at room temperature for 3 h. Control samples were incubated without lysates (without Ag). The wells of the coated plates were incubated with 200 µl of blocking buffer at 37˚C for 1 h and then with 50 µl of the bacterial lysates containing the GST fusion proteins diluted in blocking buffer (1 µg/µl total proteins) at 37˚C for 1 h. The plates were washed five times with washing buffer (0.05% Tween-20 in PBS) and the pre-incubated human sera were added and incubated at 37˚C for 1 h. After washing, the plates were loaded with donkey anti-human IgG polyclonal antibodies conjugated to horse-radish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) diluted 1:5,000 in blocking buffer, and incubated at 37˚C for 1 h. The plates were washed and stained with tetramethylbenzidine substrate (Sigma-Aldrich, Haarlem, the Netherlands) with 100 µg/ml ampicillin and 2% glucose (Sigma-Aldrich). The absorbance was measured at 450 and 630 nm. The specificity of the antibody reactions was determined by the inhibition of the reactions with the homologous antigens in comparison with the effect of the control GST protein using the formula: (Awithout Ag - Awith Ag,Ag)/(Awithout Ag - Awith GST).

Statistical analysis. Comparison of groups was performed using the Mann-Whitney test. The Spearman correlation...
**Results**

In our previous study (25), we analyzed the dataset from gene expression profiling of three subpopulations of CML stem/progenitor cells (Lin-CD34-, Lin-CD34+ and Lin+CD34+) (4). Genes with at least twice increased expression in all three subpopulations of leukemic cells in comparison to healthy controls were identified and analyzed by the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 bioinformatics tool. Twelve upregulated genes encoding centrosomal proteins (called centrosomal genes in the present study) are listed in Table II. The expression of four of them with relevance for CML as described in the Introduction (AURKA, HMMR, PLK1 and ESPL1) was analyzed in the present study.

**Expression of centrosomal genes is significantly increased at diagnosis.** Significantly enhanced expression of all four evaluated centrosomal genes in CML patients was found in total peripheral blood leukocytes at diagnosis in comparison with the level in the healthy controls (Fig. 1A). When patients were stratified based on the response to one-year treatment with IM, patients with optimal response had lower expression of the centrosomal genes at diagnosis than patients with warning or failure, but this difference was significant only for AURKA and HMMR in the patients with treatment failure (Fig. 1A).

As the expression of all studied centrosomal genes has been suggested to be upregulated by BCR-ABL1, the correlation of mRNA expression at diagnosis between centrosomal genes and BCR-ABL1 was also analyzed. However, a statistically significant correlation is shown only for AURKA (Fig. 1B).

**Delayed reduction in centrosomal-gene expression is associated with worse overall survival.** During IM treatment, peripheral blood samples were regularly taken from patients and the mRNA expression of BCR-ABL1 and centrosomal genes was quantified. We found four patterns of gene expression kinetics (Fig. 2): in pattern 1 (15 patients), the expression of centrosomal genes reached the basal level (defined by a decrease in relative expression below 2 for all genes; i.e. the mRNA levels were not twice as high as those in the controls) after three months of IM treatment and MMR was achieved within 12 months. In pattern 2 (15 patients), the expression of centrosomal genes was also quickly reduced, but MMR was not recorded within 12 months of treatment. Pattern 3, characterized by the delayed decrease in centrosomal gene expression (i.e. the basal level was not achieved within three months after IM start) and by MMR within 12 months, was found in one patient who showed MMR three months after an increase in IM dose to 600 mg/day. In 5 patients with pattern 4, the basal level of transcripts of the centrosomal genes was detected with a delay (5-9 months after IM start) and MMR was not achieved within 12 months. Both patients who died of CML showed this expression pattern.

To analyze the association of the expression of centrosomal genes with the response to IM treatment, patients were stratified into a low-risk group with a decrease in centrosomal gene expression at the first follow-up after IM start (patterns 1 and 2, 30 patients) and a high-risk group with a delayed decrease (patterns 3 and 4, 6 patients). For comparison, early molecular response (EMR) at three months was evaluated and patients were divided into a low-risk group (BCR-ABL1 >10%, 17 patients) and a high-risk group (BCR-ABL1 ≤10%, 19 patients). While EMR predicted better cumulative incidence of MMR and failure-free survival (FFS), the delayed reduction in centrosomal gene expression in the course of IM treatment was significantly associated with worse overall survival (OS) (Fig. 3).

**High overall antibody production is associated with better FFS.** In enrollment sera of 29 patients, the antibodies against AURKA, HMMR, PLK1 and ESPL1 antigens were determined by ELISA using GST-fusion recombinant proteins produced in E. coli. Due to nonspecific reactions, we did not quantify antibodies by sera titration, but the sera were incubated with antigen-carrying fusion proteins or control GST and the relative inhibition of specific reactions was calculated. For ESPL1 and PLK1, a statistically significant increase was found in comparison with sera from blood donors (Fig. 4A). After stratification of sera into low and high antibody groups based on median production, no significant differences were found for individual antigens (data not shown), but when the overall antibody production against all four antigens was
Figure 1. Centrosomal gene expression at diagnosis. (A) Relative mRNA levels in CML patients and healthy controls were compared after stratification of patients according to the molecular response to one-year imatinib mesylate (IM) treatment. Medians are indicated for groups; *P<0.05; ***P<0.001. (B) Association of centrosomal-gene transcription and BCR-ABL1 expression was examined by non-parametric Spearman's correlation analysis. CML, chronic myeloid leukemia.

Figure 2. Patterns of centrosomal gene expression after imatinib mesylate (IM) treatment. The expression of centrosomal genes and BCR-ABL1 was trimonthly determined after IM start, and expression patterns were defined in relation to the decrease in centrosomal gene expression at three months and achievement of major molecular response (MMR) at 12 months. Each graph represents gene expression in one patient. The patient with pattern 4 died 12 months after IM start.
Figure 3. Comparison of survival analysis of centrosomal gene response and early molecular response (EMR). High-risk groups were defined as the lack of normalization of centrosomal-gene expression within three months after imatinib mesylate (IM) start (patterns 3 and 4) and >10% BCR-ABL1 at three months of IM treatment, respectively. MMR, major molecular response; FFS, failure-free survival; OS, overall survival.

Figure 4. Production of antibodies against centrosomal proteins. (A) Humoral response was measured at diagnosis and compared with that of healthy controls. Medians are indicated for groups; 'P<0.05, **P<0.001. (B) Survival analysis was performed after stratification of patients into low and high antibody producers according to median production. IM, matinib mesylate; MMR, major molecular response; FFS, failure-free survival; OS, overall survival.
evaluated (means were calculated from the values of all four antigens), significantly higher cumulative incidence of MMR and FFS were shown for patients with high production of antibodies (Fig. 4B).

Discussion

As centrosomal proteins play essential roles in cell division, their expression is often increased in malignant cells. This increase can be associated with centrosome aberration leading to reduced genome stability, thus contributing to tumor progression. Our analysis (25) of the microarray dataset published by Lemoli et al (4) showed that the expression of numerous centrosomal genes was upregulated in CML progenitor/stem cells. Furthermore, a prognostic value of the HMMR overexpression has been shown for multiple myeloma (26) and B-cell chronic lymphocytic leukemia (27). Therefore, in the present study, we tested the expression of four centrosomal genes. We found markedly enhanced expression of all analyzed genes, particularly of ESPL1, at diagnosis. However, when patients with optimal response and failure were compared, only AURKA and HMMR showed a slightly significant difference in gene expression. Moreover, the probability of MMR achievement was significantly higher only in patients with low AURKA expression, and no significant difference was found for FFS (data not shown). Based on these data, we suggest that the expression of centrosomal genes in total leukocytes at diagnosis cannot be used as a prognostic marker in CML.

The follow-up of the expression of centrosomal genes during IM treatment showed that all patients achieved the basal expression. The majority of them (83%) did so in three months. As in most of the remaining patients (5/6), the delayed decrease in centrosomal gene expression was associated with treatment failure, we assigned these patients to the high-risk group. The survival analysis showed significantly reduced OS in these patients. Notably, while the three patients from this group who died during the study (two of them due to CML progression) had the BCR-ABL1 transcript level at the first follow-up after IM start reduced below 20% of that at diagnosis, the remaining three patients who survived had this level increased to >110%.

The four tested centrosomal genes have been suggested to be induced by the BCR-ABL1 protein (8,14,18,28). For AURKA, AKT signaling has been shown to be implicated in this induction (28). However, at diagnosis, we found a significant correlation with BCR-ABL1 at the mRNA level only for AURKA. Surprisingly, in all patients with therapy failure, centrosomal gene expression decreased to the basal level during IM treatment which could suggest that centrosomal gene expression can be normalized in differentiated cells in spite of resistance to IM therapy.

When humoral response to centrosomal proteins was investigated, we found that patients at diagnosis produced significantly more IgG antibodies against the ESPL1 and PLK1 antigens than healthy controls. The antibody levels did not correlate with mRNA expression of the particular gene (data not shown). Survival analysis of antibodies against individual antigens did not show significant differences, but that with the overall antibodies against all four antigens revealed a higher probability of MMR and FFS in patients with a high antibody production. IgM antibodies were not found in any of the analyzed samples and IgA antibodies did not significantly change after one-year IM treatment (data not shown).

It has been shown that IM treatment has immunosuppressive effects in CML patients that particularly influence effector T cells and dendritic cells (29). Nevertheless, immune responses during IM treatment contribute to its efficacy and can be enhanced by immunization (30). Various target antigens, including HMMR, have been identified by serological analysis (31). In the present study, the level of detected antibodies against HMMR was low, possibly due to the fact that we were able to produce only one half of the HMMR antigen for ELISA which could lack immunodominant epitope(s).

Despite the limited number of patients enrolled in the present study, our data suggest that the increased production of transcripts of centrosomal genes at CML diagnosis cannot be used in disease prognosis, but the lack of early response of centrosomal gene expression to IM therapy could be considered as a risk factor for CML progression. Furthermore, we suppose that immune responses against centrosomal proteins may contribute to the antitumor effect of IM treatment.

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