Downregulation of miR-451 in Tunisian chronic myeloid leukemia patients: potential implication in imatinib resistance

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ABSTRACT

Objectives: Resistance to imatinib has been recognized as a major challenge for the treatment of chronic myeloid leukemia (CML). Aberrant expression of miR-451 has been reported to participate in anticancer drug resistance. However, the role of miR-451 in imatinib resistance has not been investigated. The present study was undertaken to determine the expression of miR-451 in order to find a possible association between the expression of this miRNA and imatinib resistance in Tunisian CML patients.

Methods: First, real-time RT-PCR was performed to identify the expression of miR-451 in peripheral leukocytes of 59 CML patients treated with imatinib. Then, bioinformatics analysis was carried out to understand the regulatory roles of miR-451 in imatinib-resistant process.

Results: Downregulated miR-451 was observed in imatinib-resistant CML cases. In silico analysis identified MYC as a potential target of miR-451. We further revealed the existence of an MYC-binding site in MiR-451 promoter region. On the other hand, increased level of MYC was detected in imatinib-resistant CML cases which may explain the causative role of MYC in CML cases and the downregulation of miR-451.

Conclusion and discussion: Taken together, our findings suggest that miR-451 and MYC form together a regulatory loop which may act as a potential therapeutic target, and disruption of suggested regulatory loop could help to improve CML therapy.

Introduction

Chronic myeloid leukemia (CML) is a clonal myeloproliferative disorder, derived from the neoplastic transformation of the pluripotent hematopoietic stem cell [1]. CML is characterized by the generation of Philadelphia chromosome, which results from the t (9;22) (q34 q11) balanced reciprocal translocation. At the molecular level, the consequence of this translocation is the synthesis of the ‘breakpoint cluster region-Abelson murine leukemia viral oncogene homolog 1 (BCR-ABL)’ that encodes the chimeric BCR-ABL protein with constitutive tyrosine kinase activity [2]. Such a resultant protein is considered essential for the growth and survival of leukemic cells [3].

Since CML is caused by this molecular lesion, it was possible to design an efficient targeted therapy which selectively inhibits the tyrosine kinase activity of aberrant BCR-ABL protein. Imatinib mesylate (trade name Gleevec®, formerly known as STI571) is the first approved rationally designed specific inhibitor of BCR-ABL used for the treatment of CML [4]. Imatinib (IM) is a small molecule targeted therapy drug that specifically recognizes and inactivates tyrosine kinase activity of the BCR-ABL oncoprotein, thus inhibits leukemogenesis. Treatment of CML patients with IM generated excellent response, in terms of symptom control and hematological indices. However, owing to continual IM presence, primary and secondary resistance as well as molecular evidence of persistent malignancy have been observed in a significant number of CML patients [5,6]. In several cases, point mutations in the BCR-ABL kinase domain have been incremented in the development of IM resistance, secondary resistance particularly, eventually leading to treatment failure [7,8]. However, more than half of IM-resistant CML patients had no mutations in the BCR-ABL oncogene, and the basis of such BCR-ABL-independent IM resistance remains unclear [9]. Thus, it becomes difficult to predict whether a patient will develop resistance to IM.

Within the past few years, studies on miRNAs and cancer have burst onto the scene. MiRNAs are a set of small noncoding RNAs that appear to regulate the expression of coding genes in animals and plants at the post-transcriptional level. They play a key role in many physiological processes, including cell cycle, cell proliferation, apoptosis, and stem cell division [10–12]. Until recently, an increasing number of
miRNAs have been identified and linked to tumor growth [13,14] and anticancer drug resistance [15]. MiRNA profiling has become prevalent, and abundant miRNA data are currently available for various types of cancers. For instance, aberrant miRNA levels have been observed for a variety of solid tumors (Lung, breast, prostate, colon, and pancreatic cancers) [13] and hematological malignancies (B-cell lymphomas, acute lymphocytic leukemia, chronic lymphocytic leukemia, acute promyelocytic leukemia, and CML) [16,17]. These miRNAs expression signatures have been reported to be associated with diagnosis, prognosis, and response to treatment [18]. Recent studies have indicated that the pattern of miRNA expression can be correlated with anticancer drug-resistant phenotype [15], an argument further supported by reports validating miRNAs’ involvement in the tumor-cell response to chemotherapeutic agents. Among these miRNAs, miR-451 is found to play a crucial role in the pathogenesis of CML and may be involved in IM resistance [19].

In the human genome, the gene encoding miR-451 is located on chromosome 17q11.2. Such a region, 100 bp downstream of the miR-144 locus, was known to be amplified in certain types of cancers [20]. Several miRNAs expression profiling analyses have identified miR-451 for its high erythropoietic cell-restricted expression in various species, including, human [21] and mice [22]. Previous studies have demonstrated that miR-451 could inhibit cell growth [23], proliferation, and invasion and enhances apoptosis [24]. What is more, abundant evidence have established that miR-451 is widely dysregulated in human malignancies, including gastric cancer [25], glioblastoma [23], and leukemia [26]. In addition, miR-451 was found downregulated in CML cells whose expression was inversely related to BCR-ABL kinase activity, which leads to a maintenance mechanism of the leukemic state of CML cells [27]. Furthermore, transfection of miR-451 in breast cancer cells MCF-7, resistant to doxorubicin, resulted in increased sensitivity to the drug [28].

It is worth mentioning that the deregulation of genes controlled by miRNAs and the altered miRNA expression have been linked to chemotherapy resistance and progression of many disorders including cancer. Therefore, it is of particular interest to reliably predict potential miRNA targets which might be involved in drug nonsensitivity. Indeed, different large-scale genomic profiling studies and evidence have identified a large number of miRNA target genes that could play a key role in BCR-ABL independent IM resistance, including ABCB1 [29], PTPN22, TNC [30] and particularly MYC [31]. In addition, Gómez-Casares et al. showed that MYC (v-myc avian myelocytomatosis viral oncogene) deregulation may contribute to CML progression through different mechanisms including inhibition of cell differentiation [32].

All of these data suggest that miR-451 and MYC could play an important role in CML oncogenesis. However, little is known about the correlation of miR-451 and MYC with IM-resistant phenotype.

On the basis of the important role of miR-451 and MYC gene not only in the pathophysiology of CML [33], but also in drug action and development of pharmacotherapy resistance, we aimed to examine their expression profile in peripheral leukocytes of 59 newly diagnosed CML patients in order to correlate their expression with imatinib response. We also used bioinformatics analyses to better understand their regulatory roles in IM-resistant process.

**Materials and methods**

**Patient samples**

Peripheral blood samples were obtained from 59 CML patients (32 males and 27 females) in the laboratory of molecular and cellular hematology at Pasteur Institute of Tunis, Tunisia. The median age of patients with CML is 55 years. Based on major molecular remission, patients were divided into two groups according to ELN criteria 2013 (European Leukemia Net). The first group included 29 patients that are considered to be optimal IM-responders (no detectable BCR-ABL mRNA after 12 months of imatinib treatment), while the second group included 30 patients that showed IM-resistant phenotype (Therapy failure). Collection of blood samples and clinicopathologic information was undertaken with informed consent from patients. The study was approved by the ethical review approval committee of Pasteur Institute, Tunis, Tunisia.

**RNA extraction**

Peripheral blood samples were collected into tubes with EDTA. Total RNA was extracted using the miRNAeasy mini kit (reference 217004, Qiagen, Valencia, CA, U.S.A.) following the manufacturer’s protocol. RNA quality and quantity was checked using spectrophotometry. Afterward, total RNA of each sample was eluted in 30 μl nuclease-free water and stored at −80°C before use.

**Reverse transcriptase reactions**

MiR-451 was reverse-transcribed using TaqMan® microRNA RT kit (Applied Biosystems, Foster City, CA, U.S.A., #4366596) and the associated miRNA-specific stem-loop primer (TaqMan® microRNA assay kit, #4427975). Total RNA from leukocytes was diluted at a concentration of 10 ng/μl, and 5 μl of RNA was added to the reaction mix containing 0.15 μl 100 mM dNTP, 1 μl Multiscribe™ Reverse Transcriptase enzyme (50 U/μl), 1.5 μl 10 × RT buffer, 0.19 μl RNase inhibitor (20 U/μl),
3 μl RT specific-primer and 4.16 μl Rnase free water to obtain a final volume of 15 μl. RT reaction conditions were set up as follows: 30 minutes at 16°C to anneal primers, 30 minutes at 42°C for the extension of primers on miRNA and the synthesis of the first cDNA strand and 5 minutes at 85°C to stop the reaction. cDNA was then stored at −20°C until use. First-strand synthesis for real-time PCR cDNA preparation of MYC oncogene was performed on 2 μg of total RNA, using the High Capacity cDNA Reverse Transcription Kit with RNase inhibitor (Life Technologies, Foster City, CA, U.S.A., #4374966) in a final volume of 20 μl. The samples were incubated at 25°C for 10 minutes and 37°C for 120 minutes; the reverse transcriptase was inactivated at 85°C for 5 minutes and cooled at 4°C for an additional 5 minutes.

**Taqman real-time PCR**

Expression of miR-451 was analyzed using specific primers and Taqman probes according to the Taqman MicroRNA Assay protocol (Applied Biosystems-Life Technologies). Q-RT-PCR was done in a PE Applied Biosystems 7500 sequence detection system using 1.33 μl of reverse transcriptase product of miR-451 in a reaction volume of 20 μl with 10 μl TaqMan Universal PCR Master Mix, 7.67 μl of nuclease-free water and 1 μl of primer and probe mix. The reactions were incubated in a 96-well optical plate at 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. The Ct data were determined using default threshold settings. Expression of miR-451 was normalized using the expression of the housekeeping gene RNU6B. To validate mRNA levels of MYC gene identified by in silico analysis, TaqMan Gene Expression Assay (product number Hs00193527; Applied Biosystems) was used for MYC transcript quantification according to the manufacturer’s instructions, and GAPDH gene was used as the housekeeping gene for MYC normalization. All reactions were run in duplicate, and relative quantification of expression of analyzed miR-451 and MYC was calculated with the 2−ΔΔCt method.

**Statistical analysis**

The Student’s t-test was used to evaluate significant differences between two groups of data in all relevant experiments. A p-value at <0.05 (using a two-tailed unpaired t-test) was taken to indicate significant differences between two data sets. Statistical analyses and graphs were performed using GraphPad Prism version 4.03 (GraphPad Software, La Jolla, CA, U.S.A.).

**In silico analysis**

We examined the upstream miR-144/451 regions for transcription factor targets using Patch public version 1.0 algorithm (Biobase Biological Databases, http://www.gene-regulation.com/index2.html). Patch is a pattern-based program for predicting transcription factor binding sites (TFBSs) in DNA sequences. It uses the set of binding sites from TRANSFAC® Public 6.0. As miRNAs function by downregulating the expression of target genes, bioinformatics prediction of miRNA targets is important for the research of miRNA function. Therefore, we have performed a computational screen among conserved predictions for genes with complementary sites of miR-451 in their 3′-UTR using two different algorithms, Targetscan (http://www.targetscan.org/) and Microcosm (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/) hosted by the European Bioinformatics Institute. The Targetscan algorithms uses stringency in seed pairing criteria, and the conservedness of the miRNA-mRNA target pairs across vertebrate species. Apart from these two criteria in predicting target pairs, this algorithm takes into account the number of target sites for a given miRNA in a given 3′UTR, stringency in seed pairing and the parameters, which influence site accessibility. Target identification in MicroCosm is accomplished using 3′ UTRs of Ensembl transcripts and the miRanda algorithm, allowing no more than one mismatch in the critical ‘seed’ region on the 5′ end of the miRNA. Because each of the two approaches generated an unpredictable number of false positives, results were intersected to identify the genes commonly predicted by both of the algorithms.

**Results**

**Downexpression of miR-451 in imatinib-resistant CML patients**

In every case, we examined the sample for the presence of the clinically significant mutations of ABL, and no mutation was detected. We analyzed the expression profile of miR-451 by Q-RT-PCR using Taqman methodology in total leukocytes from patients with CML under IM response (n = 29) and IM-resistant (n = 30). For the comparison of miR-451 expression, data on every sample were normalized using the expression of the housekeeping gene RNU6B. Our results showed that the miR-451 was significantly downregulated in the diagnostic samples of the IM-resistant group when compared with the IM-responder group after normalization to RNU6B expression levels (p = 0.0360) (Figure 1). These data suggest that the downregulation of miR-451 could be involved in the process of imatinib resistance.

**Bioinformatic analysis for miR-451 target prediction**

MiR-451 promoter was analyzed to search TFBSs with the TRANSFAC 6.0-based algorithm; Patch 1.0. Patch
algorithm predicted the existence of an MYC-binding site located in the −1000 bp promoter region (relative to the start site of the pre-miR-144) (Figure 2). In order to determine potential genes and signaling pathways implicated in resistance to IM, we performed a computational screen for genes with complementary sites of miR-451 in their 3′-UTR using Microcosm and Targetscan algorithms. Figure 3 shows all miR-451 target genes. Many miR-451 targets have been computationally predicted, but only a limited number of these were commonly predicted by both of the algorithms. The list of predicted target genes for miR-451 can be found in supplementary file 1. The results of the bioinformatics analysis revealed that miR-451 can bind to the 3′-UTR of MYC mRNA and negatively regulate MYC expression (Figure 4). This result suggests a feedback loop between miR-451 and MYC gene bringing, therefore a balance mechanism. Such a loop, which has been defined as a Type-II circuit, exhibits bifurcating instability seeing that MYC could play a critical role in regulating the position of the ON-OFF switch at hsa-miR-451 genes products level.

**Overexpression of MYC in imatinib-resistant CML patients**

Using bioinformatics tools, we have found that MYC is a predicted target of miR-451. The expression pattern of MYC in peripheral blood samples of CML patients has not been reported. Hence, we analyzed MYC transcript level in the same cohort of patients (responders and non-responders). Q-RT-PCR was performed using cDNA synthesized from the total RNA used in the analysis of miR-451 expression. The results showed that there was statistically significant difference in the expression levels of MYC. Compared with IM-responder group, the CML resistant group displayed an increased MYC expression (*p* = 0.0341) (Figure 5), suggesting that it might contribute to imatinib resistance.

**Discussion**

In clinical setting, few molecules have been found to be associated with imatinib treatment failure for patients with CML. Thus, it is of great significance to find
novel biomarkers which can be used to explain drug resistance phenomenon of patients to chemotherapy and help to realize ‘personalized cancer medicine’ in the clinical treatment of CML. The most common mechanisms responsible for imatinib resistance are BCR/ABL kinase domain mutations. However, numerous additional mechanisms have been proposed to account for imatinib resistance in CML patients, although none have been identified as a sole source of clinical resistance leading to progressive disease [34–43]. One of the contributors to IM resistance in patients with CML is suggested to be associated with the differential expression of certain genes and miRNAs.

In the current study, our data showed that miR-451 expression level was significantly lower in imatinib-resistant CML patients compared with imatinib-responder group. In addition, using bioinformatics analysis, we found that MYC could directly bind to the promoter of miR-451 which in turn regulates the expression of MYC as a potential target gene. This suggests that miR-451 and MYC form together a feedback loop that may contribute to imatinib resistance. More importantly, we also revealed that MYC gene expression level was significantly higher in imatinib-resistant patients compared with imatinib-responder patients, which may validate the causative role of MYC in CML cases and explain the downregulation of miR-451. To our knowledge, this is the first study which demonstrated that miR-451 and MYC together may influence CML patients’ response to imatinib and may be used as candidate prognostic biomarkers for CML monitoring.

Current research is focused on the factors that contribute to cancer drug resistance, including genetic background (random drug-induced mutational events), the epigenetic mechanisms (drug-induced nonmutational alterations of gene function), and the drug-induced karyotypic changes. Because of their great importance in the regulation of gene expression, it is no doubt that miRNAs are involved in the development of chemosensitivity and chemoresistance in several types of cancer [35]. The pattern of miR-451 expression in the IM-resistant patients affecting multiple genes simultaneously provided support for this multifactorial polygenic drug resistance hypothesis.

Recently, Lopotova et al. have shown an interesting fact: the existence of a complex regulatory loop between miR-451 and BCR-ABL since miR-451 was found to be downexpressed in CML which are, at least in part, dependent on BCR-ABL activity [33]. Furthermore, in gastric cancer patients, low miR-451 expression level has been significantly correlated with worse prognosis and was shown to be embroiled in radiation therapy response [36]. Aberrant expression of miR-451 could also confer irinotecan resistance of colonspheres by decreasing expression of the ATP-binding cassette drug transporter ABCB1 and results in irinotecan sensitization in colorectal cancer stem cells [37]. In addition, increased miR-451 expression revealed that miR-451 might influence the drug resistance of the Paclitaxel-resistant breast cancer cell line [38]. Combination of all those findings suggests that miR-451 could play a major role in CML-IM resistance which explains, at least in part, the variable responses to this drug. Since aberrant expression of miR-451 was significantly correlated with the development and progression of CML, reversing the altered miR-451 may become a hopeful strategy for the prevention and treatment of CML.

Since each miRNA has hundreds of different conserved or non-conserved targets, the alterations in the level of a miR-451 by altered MYC expression could cause significant alterations in the expression of many target genes, and thereby promote aberrations in multiple cellular signal transduction pathways, resulting in the progression of CML and development of IM resistance. To obtain insight into the potential function of miR-451 in the process of IM resistance, we further focused on the function of putative target genes based on in silico methods. Accordingly, we revealed that MYC is a potential target gene of miR-451. A number of previous reports have been focused on the role of MYC gene on cell proliferation, differentiation and genomic instability, while its role in drug resistance especially IM resistance has not been fully elucidated. As a potential transcription factor, MYC was found to directly or indirectly regulate about 1000 genes, and binds to 15% of genomic loci [39,40]. Porro et al. demonstrated that MYC could transcriptionally upregulate the ATP-binding cassette
transporter genes in CML CD34+ progenitors cells which is consistent with the idea that leukemia stem cell is resistant to the therapy [41]. On the other hand, Chang et al. suggest that the predominant consequence of activation of MYC gene is widespread repression of several miRNA expressions [42]. Here, using bioinformatics analysis, we found that MYC could directly bind to the promoter of miR-451 which can explain the downregulation of miR-451 in CML-IM-resistant patients by the consequent upregulation of MYC gene.

On the other side of the spectrum, during our bioinformatics analysis, we found that miR-451 can be directly associated with the 3′-untranslated region of the MDR1 (ABCB1) mRNA and could inhibit its expression. IM is a substrate for ABCB1 transporter. Hence, ABCB1 might be influencing pharmacokinetics and intracellular or systemic levels of IM and contribute to resistance by extruding IM from hematopoietic cells. Recently, there have been several reports that intracellular levels of IM are influenced by the upregulation of the efflux transporter ABCB1 [43]. Such a finding could be explained by the consequent downregulation of miR-451 in IM-resistant CML cases. Taken together, all these findings suggest that targeting miR-451 could provide an efficient approach for the prevention or treatment of CML. In summary, we have shown that the development of imatinib resistance in CML patients is associated with the pronounced deregulation of miR-451 expression. Additionally, we have shown that expression of MYC gene, a potential target of miR-451, is upregulated in IM-resistant CML patients. These results provide a strong rationale for the development of miRNA-based therapeutic strategies aiming to overcome imatinib resistance in CML patients. However, these alterations are not necessarily indicative of the causative role of miR-451 deregulation in the CML’s imatinib resistance development, and the ultimate goal of future studies is to address this question.

Disclosure statement
No potential conflict of interest was reported by the authors.

Ethical standard
All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

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