ORIGINAL ARTICLE

CORRELATION BETWEEN THE EXPRESSION OF ARGININO-SUCCINATE SYNTHETASE GENE AND DRUG RESISTANCE MECHANISM OF CHRONIC MYELOGENOUS LEUKAEMIA

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Abstract

Chronic myelogenous leukaemia (CML) induced by malignant clone of hematopoietic stem cell is usually treated with imatinib mesylate. The treatment with imatinib has two main problems, first it cannot thoroughly eliminate leukaemia stem cells and second it develops primary or secondary drug resistance. This study investigated whether the gene deletion of argininosuccinate synthetase (ASS) was the reason for the drug resistance to CML and analysed their relationship as well as the clinical effect of imatinib in treating CML. Bone marrow samples of CML patients were collected. Sample analysis was performed using fluorescence *in situ* hybridization (FISH) of bone marrow cell. Fifteen out of one hundred patients with CML were found to be lacking ASS gene, among which 11 patients received imatinib treatment. 10 of these patients were in chronic phase and survived and one patient was in acceleration phase and survived. 40% (4 patients) from those in CP registered disease progress and 1 patient from those in AP had progress. Among the 62 patients without the deletion of ASS who received imatinib treatment, the disease condition of only 12.9% (8 patients) showed progress, suggesting that the deletion of ASS gene could produce drug resistance in the treatment of chronic myelogenous leukaemia. It can be concluded that, the deletion of ASS gene can affect the curative effect of imatinib in treating CML and can promote the progress of the disease. Therefore, it can be used for evaluating the prognosis of patients.

Rezumat

Leucemia mieloidă cronică (LMC) indusă de clone maligne ale celulelor stem hematopoietice este tratată de obicei cu imatinib mesilat. Tratamentul cu imatinib prezintă două probleme importante: în primul rând nu poate elimina în totalitate celulele stem leucemice şi în al doilea rând în timpul tratamentului poate apărea rezistenţă primară sau secundară la medicament. Acest studiu a urmărit legătura dintre depleţia genei argininosuccinat sintetazei şi rezistenţa la medicamente, precum şi efectul clinic al imatinibului în LMC. Au fost recoltate probe de măduvă osoasă de la pacienţi cu LMC. Analiza probelor s-a realizat prin hibridizare de fluorescenţă *in-situ* (FISH) a celulelor provenite din măduvă osoasă. 15 din cei 100 de pacienţi cu LMC au prezentat lipsa genei argininosuccinat sintetazei, dintre care 11 au fost trataţi cu imatinib. 10 dintre aceştia s-au aflat în faza cronică (FC) şi au supravieţuit şi 1 pacient s-a aflat în faza accelerată (FA) şi a supravieţuit. Boala a progresat la 40% (4 pacienţi) dintre cei cu FC şi la 1 pacient dintre cei cu FA. Numai 8 din cei 62 de pacienţi fără depleţia genei argininosuccinat sintetazei, care au primit tratament cu imatinib, au prezentat progresia bolii, ceea ce sugerează faptul că rezistenţa la tratament în LMC poate fi dată de depleţia genei argininosuccinat sintetazei. În concluzie, depleţia genei argininosuccinat sintetazei influenţează efectul curativ al imatinibului în LMC şi influenţează progresia bolii. În plus, determinarea mutaţiei acestei gene poate fi utilizată pentru evaluarea prognosticului acestor pacienţi.

Keywords: argininosuccinate synthetase gene; chronic myelogenous leukemia; imatinib; clinical effect

Introduction

Chronic myelogenous leukaemia (CML) induced by malignant clone of hematopoietic stem cell [4] is manifested as splenomegaly, continuous increase of peripheral blood leucocyte, positive fusion of Philadelphia chromosome (Ph) and BCR/ABL gene [21]. Ph can be used as an important basis for diagnosis of CML. Zhang L. *et al.* [18] studied the acute lymphoblastic leukaemia (ALL) with positive H chromosome and achieved great progress by using imatinib treatment. BCR gene was located on the long arm of the chromosome 22, which had 23 exons and 160 protein products. The ABL gene was situated on the long arm of the chromosome 9, which had 12 exons. The main structure included sarcoma homologous SH2 and SH1. Al Achkar W. *et al.* [1] believed that such fusion gene played a potential role in the progress of CML. Tyrosine kinase (TK) encoded by ABL gene can lead to cell proliferation, enhance cell transformation, weaken the dependency of the growth factor, and promote the ability of leukaemia cells to resist apoptosis, leading to its excessive multiplication, and thus

result in the occurrence of CML [12]. CML can be typed into chronic phase, acceleration phase and blast phase. In chronic phase, there are no symptoms; three to five years later, the acceleration phase arrives, being accompanied by symptoms such as hyperpyrexia, bleeding and arthralgia; six to nine months later, blast phase arrives together with extremely high death rates [6, 14].

CML is usually treated with imatinib mesylate. Imatinib, a BCR-ABL tyrosine kinase inhibitor, interfere with intracellular signalling pathways and inhibit carcinogenesis pathways, being used with success to treat different types of cancers since it was first introduced on the market in 2001 [2]. All the tyrosine kinase inhibitors have showed better tolerability profile and less toxicity compared with conventional cytotoxic drugs [10], and because of its favourable effect and high tolerance, imatinib gradually becomes the first-line scheme in the initial treatment of CML [13]. Jiang B. showed that, the genetic and molecular effects on patients with Ph-positive CML were significantly improved after treatment using imatinib mesylate [7]. A big problem in the treatment with imatinib mesylate is that it cannot thoroughly eradicate leukaemia stem cells and develops primary or secondary drug resistance [15]. Argininosuccinate synthetase (ASS) gene, which is close to ABL gene, is distributed on 9q34.1 chromosome [13]. Dong J. *et al.* found that, ASS gene is absent in 10 - 30% of patients with CML [3]. Huntly B.J. *et al.* thought that, the deletion of fracture site from 9q34.1 chromosome was correlated to the effect of imatinib [5]. Whether the deletion of ASS gene can result in drug resistance to imatinib has not been proved yet. Thus, this study aims to detect the expression of ASS gene among CML patients, and to provide a scientific support for the drug resistance of CML to imatinib.

Materials and Methods

Patients

100 patients with CML who received treatment in the Tumour Hospital of Shanghai, Fudan University, China, from January 2013 to January 2016 were selected. The dose of imatinib used was 400 mg/day in chronic phase and 600 mg/day in acceleration phase. When the count of blood platelet and white blood cells was normal, there were no immature metrocyte and bone marrow cells in peripheral blood, and when there was no obvious extra-marrow leukaemia signs such as splenomegaly, it was considered as complete hematologic response; it was considered as major cytogenetic response (MCyR) when Ph positive cells = 1 - 35%; it was considered as complete cytogenetic response (CCyR) when Ph positive cells = 0 and Ph chromosome turned negative. Major cytogenetic remission was determined by standard cytogenetic analysis in 20 metaphases. Major molecular response was defined as a BCR-ABL1/ABL1 transcript ratio of lower than 0.05% by QRT-PCR, representing more than 3-log reduction from the baseline for untreated patients in our laboratory. Complete molecular response was defined as undetectable levels of BCR-ABL1 transcript, representing at least 4.5 - log reduction from baseline levels [19].

All the experiments have been approved by the medical ethics committee and verified by pathologists. Patients signed the informed consent before enrolling in the study.

Reagents and equipment

Materials and equipment included ethyl alcohol (70%, 85% and 100%) (Shanghai Xinfan Biotech. Co., Ltd., China), KCl (Shanghai Xinfan Biotech Co., Ltd., China), methyl alcohol (Huanghua Pengfa Chemical Co., Ltd., China), glacial acetic acid (Huanghua Pengfa Chemical Co., Ltd., China), pepsin (Ningxia Xiasheng Group, China), BCR/ABL1 dual-colour dual-fusion fluorescence *in situ* hybridization (FISH) probe (Hongkong Huachuang Trading International Co., Ltd., China), 4',6-diamidino-2-phenylindole (DAPI) (Shanghai Yeasen Biotech. Co., Ltd., China), NP-40 (Jiangsu Haian Petrochemical Plant, China), water bath (Tianjin Binhe Trading Co., Ltd., China), staining jar (Tianjin Binhe Trading Co., Ltd., China), 37°C incubator (Tianjin Binhe Trading Co., Ltd., China), fluorescence microscope (Guangzhou Mingmei Guangdian Technology Co., Ltd., China), hybrid wet box (Shanghai Suolaibao Bio-technology Co., Ltd., China), centrifuge (Zhengzhou Nanbei Instrument Equipment Co., Ltd., China), and FISH analysis software.

Solutions

Firstly, 20 × saline sodium citrate (SSC) solution (pH = 5.3) was prepared by adding 500 mL of double distilled water into 87.5 g of sodium chloride and 44.1 g of sodium citrate. Then 2 × SSC solution was prepared by adding 450 mL of double distilled water into 500 mL of 20 × SCC solution, and the pH value was adjusted to 7 using sodium hydroxide. Next, 0.4 × SSC solution was prepared by adding 490 mL of distilled water into 10 mL of 20 × SSC solution, and the pH value was adjusted to 7 using sodium hydroxide. 2 × SSC/0.1% NP-40 was prepared by adding 850 mL of purified water and 3 mL of NP-40 into 100 mL of 20 × SSC (pH = 5.3) and stored at 5°C after its pH value was adjusted to 7 using sodium hydroxide.

0.4 × SSC/0.3%NP-40 was prepared by adding 950 mL of purified water and 3 mL of NP-40 into 20 mL of 20 × SSC (pH = 5.3) and stored at 5°C after its pH value was adjusted to 7. 49 mL of formamide, 7 mL of 20 × SSC (pH = 5.3) and 14 mL of purified

water were mixed up and stored with cover at 5°C after the pH value was adjusted to 7. 105 mL of formamide, 21 mL of 20 × SSC (pH = 5.3) and 84 mL of purified water were mixed up and stored with cover at 5°C after the pH value was adjusted to 7.5.

Sample processing

1 - 3 × 10^6/mL of bone marrow which had been processed by heparin anticoagulant was added into 10 mL of 0.075 M KCl solution. The solution was blew and mixed lightly, stored in 37°C water tank, and taken out after 30 min of low permeability.

Afterwards 1 mL of methyl alcohol and 10 mL of glacial acetic acid fixer (3:1) were added in the solution and centrifuged at 1000 r/min for 10 min. After the supernatant was removed, the cells precipitated. Then 10 mL of fixer was added. After 20 min of placement at room temperature, the solution was centrifuged at 1000 r/min for ten minutes. The third step was repeated twice. The supernatant was removed and the cells precipitated. After the addition of the fixer, the cell suspension was stored at -20°C.

Experimental procedures

The collected samples were dropped on a glass sheet. The quality of cells was observed under a microscope. Samples with evenly distributed cells, sufficient quantity and without or with small amounts of cytoplasm were selected. Moreover, the hybrid area was recorded. The sections were incubated at 50°C for two hours.

50 mg of pepsin, 1 mL of purified water and 365 mL of diluted hydrochloric acid were mixed evenly. Then it was dropped on a glass sheet using a dropper for one minute of reaction. 2 × SSC solution, 2 × SSC/0.1% NP-40 solution and ethyl alcohol (70%, 85% and 100%) were processed by dehydration for 3 minutes.

7 μL of hybridization solution and 3 μL of probe were put into a 0.5 mL tube, and centrifuged for 3 s. The hybrid area was delimited on the glass sheet. 10 μL of mixed liquor was put in the area, covered by a 19 mm × 19 mm cover glass, and mounted. The glass slide was put on a constant temperature thermal platform (70°C).

The glass sheet and probe were degenerated at the same time point. Two minutes later, the glass sheet was hybridized overnight in a constant-temperature incubator (37°C).

The sealed glass sheet was transferred into 0.4 × SSC/0.3% NP-40 (73°C) solution and taken out after 2 min of washing.

Further the glass sheet was washed by 2 × SSC/0.1% NP-40 solution for 1 minute; it was transferred with 10 μL of DAPI to the hybrid area and sealed up.

Two hundred interphase nucleuses of each glass sheet were randomly analysed and the signals were recorded.

Statistical analysis

The data were statistically analysed using SPSS ver. 17.0. The comparison between groups was performed by Wilcoxon rank test. The cytogenetical effect was processed by Fisher test. $p < 0.05$ was considered statistically significant.

Results and Discussion

Through sample processing, the following data were obtained. One hundred patients were included in this experiment, 62 patients were male and 38 patients were female. Besides, 91 patients were in chronic phase and 9 patients were in acceleration phase. Figure 1 shows that, fifteen patients were detected with the deletion of ASS, accounting for 15%. 11 patients in the deletion group (73.3%) and 62 patients in the non-deletion group (72.9%) were treated with imatinib. Statistical processing using SPSS ver. 17.0 suggested that the difference between the two groups had no statistical significance ($p > 0.05$).

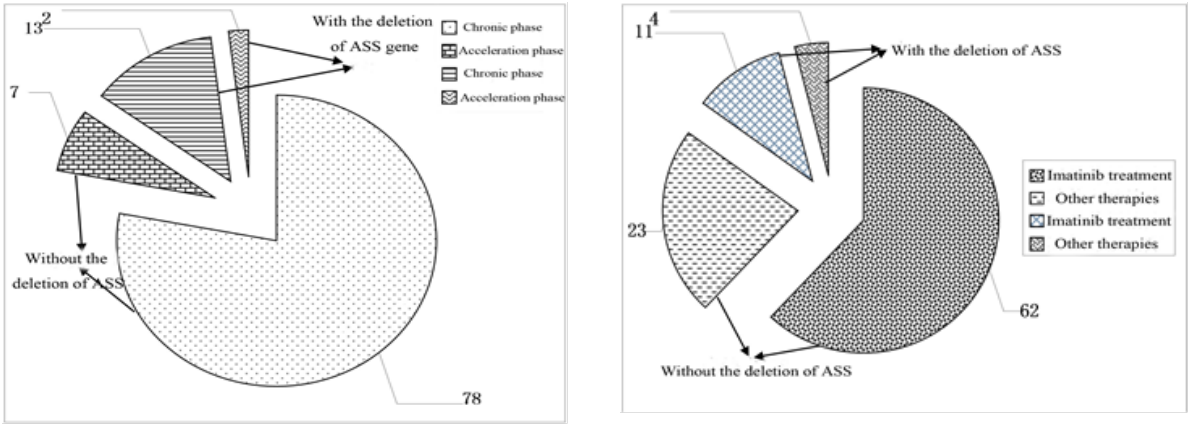

Figure 1.
The statistics of ASS gene deletion conditions in different phases and related therapies

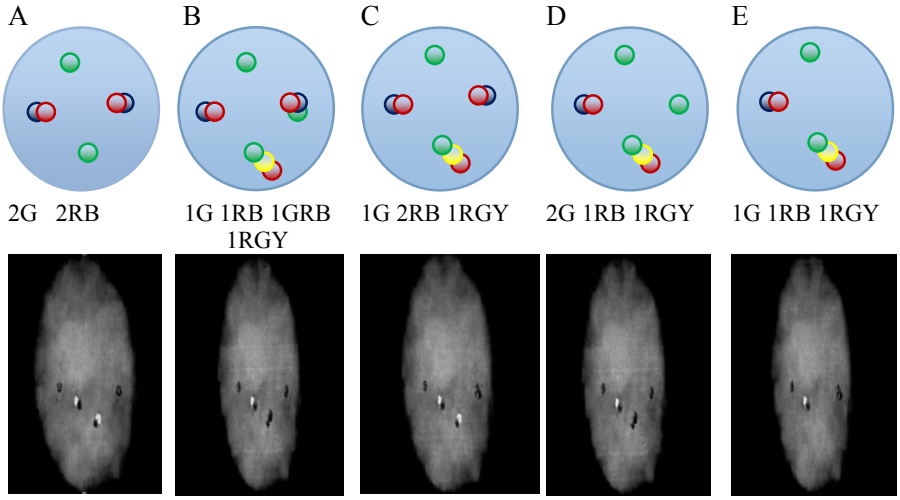

Figure 2.
Chromosome under probe
Note: green: chromosome 22; red and blue: chromosome 9; yellow: Ph chromosome

Chromosomes of the patients were detected using probes and several situations occurred. Figure 2A demonstrates the model of chromosome 22 and 9 in normal condition; Figure 2B suggests a signal model without deletion during typical Ph transposition; Figure 2C suggested a model with chromosome 22 signal deletion in the derivative of chromosome 9; Figure 2D shows a model with ASS deletion on chromosome 9; Figure 2E demonstrates a model with BCR deletion and ASS deletion in chromosome 22. Figures 2A, 2B, 2C, 2D and 2E were in correspondence with the above probe images respectively. Chromosome detection could easily discover gene deletion as well as the deletion of ASS gene, which was beneficial for a better treatment.
The effect of imatinib on patients with ASS gene deletion is shown in Tables I and II.

Table I
The disease progress and survival condition of patients in acceleration and chronic phases who were treated by imatinib

Disease phase	Imatinib treatment	Number of survived patients	Number of patients with disease progress	Proportion	p value (compared with patients without the deletion of ASS who received imatinib treatment and suggested disease progress)
Chronic phase	10/11	10/10	4/10	45.5%	< 0.05
Acceleration phase	1/11	1/1	1/1		

Table II
Cytogenetic effect of imatinib in acceleration and chronic phases

Phase	Complete hematologic remission	Major cytogenetic remission	Complete cytogenetic remission
Chronic phase	0/10	4/10	6/10
Acceleration phase	0/1	1/1	0/1

As shown in Table I, among the 11 patients with ASS gene deletion who received imatinib treatment, 10 patients were in chronic phase and survived, and one patient was in acceleration phase and survived. 4 patients among those in chronic phase and 1 patient among those in acceleration phase had progress. The number of patients with progress was 45.5% that of the patients who had the deletion of ASS and received imatinib treatment. Among the patients without the deletion of ASS who received imatinib treatment, only 8 patients showed disease progress, which was 12.9% that of patients without the deletion of ASS who received imatinib treatment. The difference of proportion between the two groups suggested statistical significance ($p < 0.05$). It indicated that, patients with the deletion of ASS who received imatinib treatment are more likely to register disease progress. Table II demonstrates that, 4 out of 10 patients who received imatinib treatment showed MCyR, 6 patients showed CCyR, and one patient in acceleration phase showed MCyR as well. The proportion of patients with MCyR was 45.5%, while that of patients with CCyR was 54.5%. In the group of patients without the deletion of ASS, sixty-two patients received imatinib treatment, among which, the proportion of patients with MCyR was 35.5% and the proportion of patients with CCyR was 64.5%; the difference showed no

statistical significance ($p > 0.05$). Among 100 patients, 73 patients were treated with imatinib all the time; 10 patients in the deletion group and 63 patients in the non-deletion group were treated for more than three months. Among the patients whose treatment course lasted for more than 12 months and showed complete cytogenetic remission, 5 were from the deletion group and 35 were from the non-deletion group, suggesting drug resistance was generated in the deletion group.

Ph chromosome, the genetic hallmark of CML cells, is formed by the balanced translocation of ABL protooncogene on chromosome 9 and the genes on chromosome 22 [8]. When the breaking point appears, the sequences of chromosome 9 and 22 may fracture [16]. It has been found that, the deletion of the surrounding fracture site fragments of derivative chromosome 9 is one of the reasons for poor prognosis of leukaemia [17]. Traditional FISH technology is lowly sensitive to the deletion of ASS [20]; therefore, BCR/ABL1 dual-colour dual-fusion FISH technology was adopted to investigate the deletion of ASS of CML patients. It was found that, most of the patients were in chronic phase and few were in acceleration phase. As to whether the deletion of ASS can influence the effect of imatinib treatment on CML patients with ASS deletion, Soenen *et al.* considered that, the deletion of fracture segments in derivative chromosome 9 had no influence on the effect of imatinib treatment in the treatment of CML [11]. This study found that, the effect of imatinib was in correlation to the deletion of ASS. Moreover, it was found that, the deletion of ASS gene had a great influence on disease progress, and the progress conditions of the deletion group and the non-deletion group suggested a statistically significant difference. It was assumed that, the deletion of ASS gene resulted in the unstable structure of chromosome, which produced larger influence on the disease progress. But in the aspect of curative effect, the effect of the deletion group was not as obvious as that of the non-deletion group due to the generation of drug resistance.

Conclusions

The patients with the deletion of ASS gene could not obtain the curative effect the same as the patients without deletion. Compared to the other treatment methods, imatinib showed a good genetic cellular reaction to the patients with the deletion of ASS gene, suggesting imatinib influenced the treatment progress of MCL. Due to the limited knowledge, the specific influence on imatinib remains to be further investigated. The deletion of ASS gene has certain influence on the effect of imatinib and can promote disease progress. Appropriate adjustment must be carried out rapidly if the expected therapeutic effect can't be achieved.

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