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Time-series Analysis in Imatinib-resistant Chronic Myeloid Leukemia K562-cells under Different Drug Treatments*

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Summary: Chronic myeloid leukemia (CML) is characterized by the accumulation of active BCR-ABL protein. Imatinib is the first-line treatment of CML; however, many patients are resistant to this drug. In this study, we aimed to compare the differences in expression patterns and functions of time-series genes in imatinib-resistant CML cells under different drug treatments. GSE24946 was downloaded from the GEO database, which included 17 samples of K562-r cells with (*n*=12) or without drug administration (*n*=5). Three drug treatment groups were considered for this study: arsenic trioxide (ATO), AMN107, and ATO+AMN107. Each group had one sample at each time point (3, 12, 24, and 48 h). Time-series genes with a ratio of standard deviation/average (coefficient of variation) >0.15 were screened, and their expression patterns were revealed based on Short Time-series Expression Miner (STEM). Then, the functional enrichment analysis of time-series genes in each group was performed using DAVID, and the genes enriched in the top ten functional categories were extracted to detect their expression patterns. Different time-series genes were identified in the three groups, and most of them were enriched in the ribosome and oxidative phosphorylation pathways. Time-series genes in the three treatment groups had different expression patterns and functions. Time-series genes in the ATO group (e.g. *CCNA2* and *DAB2*) were significantly associated with cell adhesion, those in the AMN107 group were related to cellular carbohydrate metabolic process, while those in the ATO+AMN107 group (e.g. *AP2M1*) were significantly related to cell proliferation and antigen processing. In imatinib-resistant CML cells, ATO could influence genes related to cell adhesion, AMN107 might affect genes involved in cellular carbohydrate metabolism, and the combination therapy might regulate genes involved in cell proliferation.
Key words: chronic myeloid leukemia; time-series genes; expression pattern; AMN107 and ATO combination

Chronic myeloid leukemia (CML) is a hematopoietic stem cell disorder characterized by positive Philadelphia chromosome (Ph), which leads to the formation of active BCR-ABL fusion protein[1, 2]. Based on a recent epidemiological data, the annual incidence of CML in Europe was approximately 10 per million persons, with a median age at diagnosis of 57–60 years[3].

BCR-ABL tyrosine kinase inhibitors (TKI) are effective in the treatment of CML. Imatinib, a TKI, was identified as the first-line treatment of CML, and it has been shown to increase the response and survival rates remarkably[4]. However, the efficacy of imatinib is limited because many patients with CML are resistant or intolerant to this drug[5]. Therefore, new drugs should be developed to improve CML prognosis, especially in imatinib-resistant patients. Dasatinib is a potent BCR-ABL TKI, has a higher inhibitory activity than imatinib, and it is effective in patients with imatinib-resistant CML[6]. AMN107 (also known as nilotinib), another highly selective BCR-ABL TKI, is effective in patients with imatinib-resistant/intolerant CML[7]. Both the above-mentioned TKIs function by targeting the BCR-ABL protein. However, in patients with BCR-ABL-independent imatinib resistance, they do not provide desirable health benefits.

Several factors that are crucial for the maintenance of CML stem cells, such as promyelocytic leukemia (PML), have been identified. The inhibition of these factors in combination with TKI agents could successfully eradicate TKI resistance in CML stem cells[8]. Arsenic trioxide (ATO), an anticancer agent, when used in combination with cytarabine (Ara-C) has been proven to decrease the expression of PML[8]. The synergistic effect between ATO and AMN107 on imatinib-resistant CML K562-r cells via induction of endoplasmic reticulum stress-mediated apoptosis has been demonstrated[9]. Recently, it was reported in a study that the combination of the aforementioned drugs inhibited cell proliferation in patients with CML in the blast crisis phase[10].

Despite providing valuable findings, the previous studies did not focus on the simultaneous comparison of molecular changes among ATO, AMN107, and their combination treatments. The identification of time-series genes helped to uncover the mechanisms underlying transcriptional regulation[11]. In the present study, we re-analyzed the GSE24946 dataset, established by Xia *et al*. and containing CML samples at different time points during the three treatments (ATO, AMN107, and combi-

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nation therapy)[9], to compare the gene expression patterns under the three treatments. Moreover, through functional and pathway enrichment analyses, we intended to screen potential gene markers for specific functions or pathways to obtain better guidelines for targeted therapy of CML with the three treatments respectively.

1 MATERIALS AND METHODS

1.1 Data Pre-processing and Screening of Time-series Genes

The transcription profile of GSE24946[9] was downloaded from the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) database, which was based on the GPL11118, HIS/SIBS Human 15K cDNA array platform. The samples in this dataset included K562-r cells with (n=12) or without drug administration (n=5). The cells were treated with 1.5 μmol/L ATO (ATO group), 8 μmol/L AMN107 (AMN107 group), and their combination (ATO+AMN107 group), and cultured for 3, 12, 24 and 48 h, respectively. One sample was present in each group at each time point.

Raw data for expression profiling were preprocessed using the R software in Bioconductor (http://www.bioconductor.org/)[12]. First, the probes were converted into gene symbols, and the non-expressed probes were filtered out. If multiple probes corresponded to the same gene, their values were averaged to obtain the gene expression. Then, the average value and standard deviation (SD) of gene expression at four time points were calculated. In each group, the genes with a ratio of SD/average value greater than 0.15 were defined as time-series genes[13].

1.2 Pathway Enrichment Analysis and Clustering of Expression Patterns

The online tool, Database for Annotation, Visualization, and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov/)[14] was employed to perform the pathway enrichment analysis of time-series genes based on the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/pathway.html) database. The raw P-values were adjusted to control the false discovery rate (FDR) by using the Benjamini-Hochberg (BH) method[15], and an FDR less than 0.05 was considered as the cut-off for significant pathway categories.

The Short Time-series Expression Miner (STEM) software was used to cluster genes with coherent changes[16]. A P-value <0.05 was set as the threshold for significant expression pattern. Afterward, the different expression patterns of time-series genes under the three treatment groups were compared.

1.3 Functional Enrichment Analysis

The Gene Ontology (GO, http://www.geneontology.org/) enrichment analysis has become the most common approach for functional annotation of large-scale genomic data[17]. Functional enrichment analysis of time-series genes was carried out to identify significant gene expression patterns, and the GO functional categories were considered significant if the P value was less than 0.05. The top ten significant GO categories were identified, and time-series genes in these categories were extracted to determine their expression patterns.

2 RESULTS

2.1 Identification of Time-series Genes

Based on the aforementioned method, the time-series genes in leukemia K562-r cell lines were identified in the three different treatment groups: 1847 genes in ATO group, 1796 genes in AMN107 group, and 1641 genes in ATO+AMN107 group. The Venn diagram shows that 1377 time-series genes overlapped in the three treated groups (fig. 1).

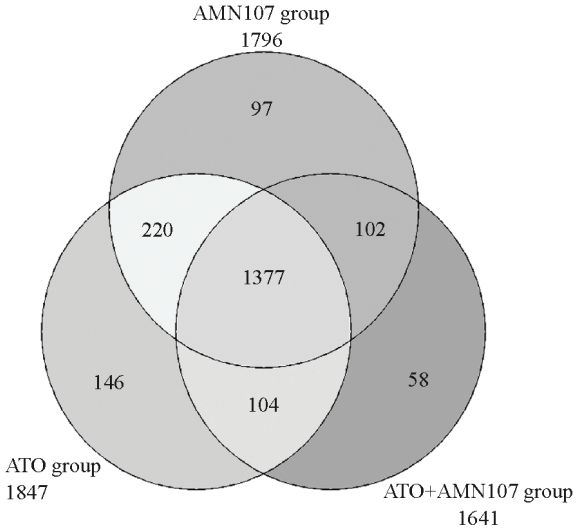

Fig. 1 Venn diagram of time-series genes in chronic myeloid leukemia K562-r cells under different drug treatments

2.2 Pathway Enrichment Analysis of Time-series Genes

The pathways with an FDR less than 0.05 were considered significant. From table 1, we found that the time-series genes in the three groups were enriched in similar pathways, such as the ribosome and oxidative phosphorylation pathways.

2.3 Expression Pattern Clustering of Time-series Genes

A comparison of the expression patterns of time-series genes revealed that they were different among the three groups. Notably, many time-series genes in the ATO and AMN107 groups had similar expression patterns (e.g. genes in profile 29, 39, 43, 49, 38, and 41); however, similar expression patterns were not found among the three groups. In particular, the gene expression patterns in profile 29 and 49 in the ATO+AMN107 group were markedly different from those in the other two groups. In addition, the gene expression pattern in profile 40 was significantly different from that in the ATO group. The time-series genes in profile 29 showed a stable-increasing-stable expression trend in ATO+AMN 107 group, whereas they presented a stable-increasing-decreasing expression trend in the other two groups. In profile 49, time-series genes showed an increasing-decreasing expression trend in ATO+AMN107 group, whereas they exhibited an increasing-stable-increasing-decreasing trend in the other two groups. In profile 40, time-series genes presented an increasing-stable-increasing expres-

sion trend in ATO+AMN107 group, whereas they exhibited an increasing-decreasing trend in the ATO group (fig. 2, tables 2 and 3).

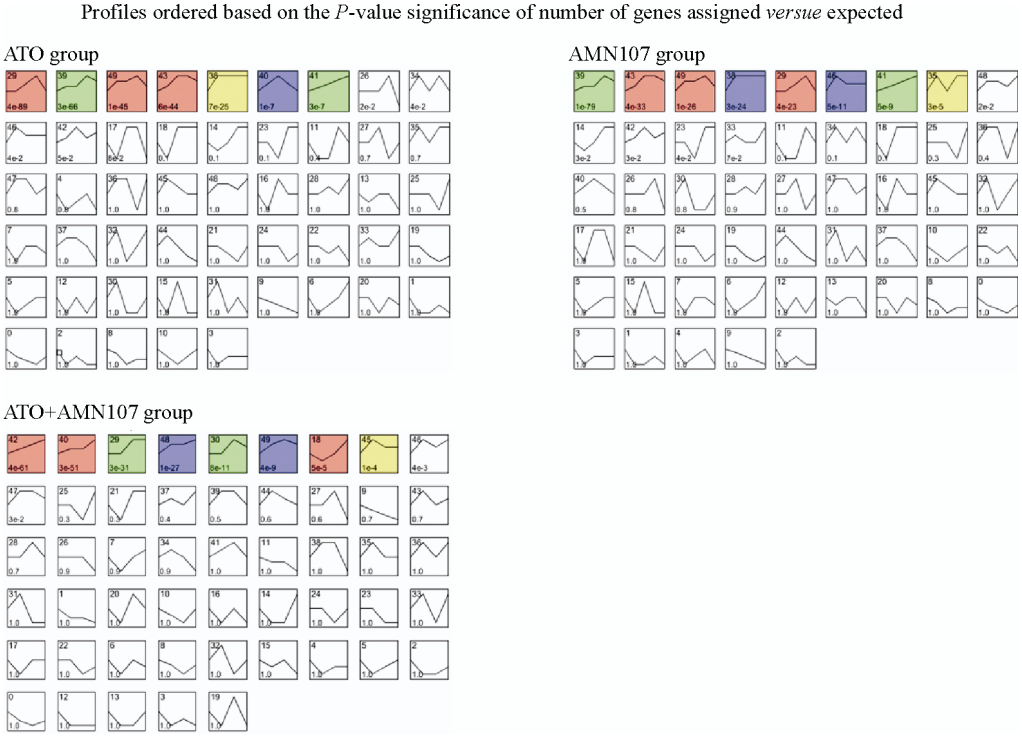

Fig. 2 Expression patterns of time-series genes in chronic myeloid leukemia K562-r cells under different drug treatments
The colored profiles represent significant profiles.

Table 1 Pathway enrichment analysis of time-series genes

Groups	KEGG pathway	Gene count	Bonferroni *P*-value	FDR
ATO	hsa03010:Ribosome	49	7.47E-18	5.00E-17
	hsa00190:Oxidative phosphorylation	57	2.05E-14	1.33E-13
	hsa05012:Parkinson's disease	54	1.01E-12	6.73E-12
	hsa05016:Huntington's disease	64	3.13E-11	2.09E-10
	hsa05010:Alzheimer's disease	53	2.21E-07	1.48E-06
	hsa03050:Proteasome	20	9.99E-04	6.68E-03
	hsa03040:Spliceosome	36	4.03E-03	2.70E-02
AMN107	hsa03010:Ribosome	47	9.52E-17	6.40E-16
	hsa00190:Oxidative phosphorylation	50	1.83E-10	1.23E-09
	hsa05012:Parkinson's disease	48	1.62E-09	1.09E-08
	hsa03050:Proteasome	26	1.17E-08	7.86E-08
	hsa05016:Huntington's disease	55	3.09E-07	2.08E-06
	hsa05010:Alzheimer's disease	48	1.45E-05	9.73E-05
	hsa03040:Spliceosome	38	2.23E-04	1.50E-03
ATO+AMN107	hsa03010:Ribosome	50	4.59E-21	3.08E-20
	hsa00190:Oxidative phosphorylation	43	1.73E-07	1.16E-06
	hsa03050:Proteasome	22	5.34E-06	3.59E-05
	hsa05012:Parkinson's disease	39	1.47E-05	9.90E-05
	hsa03040:Spliceosome	38	2.99E-05	2.01E-04
	hsa05016:Huntington's disease	46	2.36E-04	1.59E-03

KEGG: Kyoto Encyclopedia of Genes and Genomes; FDR: false discovery rate

Table 2 The statistics of time series genes' profiles in different groups

Groups	Significant profile number	Cluster number
ATO	7	4
AMN107	8	4
ATO+AMN107	8	4

Table 3 Overlapped profiles of time series genes in different drug treatments

Drug treatments	Profile ID
ATO, AMN107 and ATO+AMN107	29, 49
ATO and AMN107	39, 43, 38, 41
ATO and ATO+AMN107	40
AMN107 and ATO+AMN107	–
ATO	–
AMN107	46, 35
ATO+AMN107	42, 48, 30, 18, 45

ID: identity

2.4 Functional Enrichment Analysis of Time-series Genes in Different Expression Patterns

The top ten GO categories of time-series genes were selected based on GO enrichment analysis. Then, the expression patterns of time-series genes enriched in the ten GO categories were identified (table 4). It was observed that in the ATO group, the enriched functional categories associated with positive regulation of cell adhesion, and negative regulation of DNA binding had the same expression pattern as profile 40, which exhibited an increasing-decreasing trend. In addition, time-series genes enriched in the cell-cell adhesion GO categories exhibited the same expression pattern as profile 38, which showed an increasing trend initially and then remained stable (table 4).

Table 4 The distribution of the top 10 GO categories in different expression patterns

Groups	Category ID	Category name	Uncorrected *P*-value	Profile ID
ATO	GO:0045785	Positive regulation of cell adhesion	1.10E-05	40
	GO:0030155	Regulation of cell adhesion	1.30E-04	40
	GO:0016337	Cell-cell adhesion	2.80E-04	38
	GO:0008236	Serine-type peptidase activity	4.40E-04	39
	GO:0017171	Serine hydrolase activity	4.40E-04	39
	GO:0006869	Lipid transport	8.30E-04	38
	GO:0043392	Negative regulation of DNA binding	1.10E-03	40
	GO:0010811	Positive regulation of cell-substrate adhesion	1.40E-03	40
	GO:0051101	Regulation of DNA binding	1.40E-03	40
	GO:0032731	Positive regulation of interleukin-1 beta production	1.90E-03	41
AMN107	GO:0070011	Peptidase activity, acting on L-amino acid peptides	2.00E-04	39
	GO:0004725	Protein tyrosine phosphatase activity	2.70E-04	35
	GO:0034707	Chloride channel complex	3.10E-04	39
	GO:0008233	Peptidase activity	6.30E-04	39
	GO:0042578	Phosphoric ester hydrolase activity	6.60E-04	35
	GO:0010677	Negative regulation of cellular carbohydrate metabolic process	1.10E-03	29
	GO:0008608	Attachment of spindle microtubules to kinetochore	1.20E-03	35
	GO:0051988	Regulation of attachment of spindle microtubules to kinetochore	1.20E-03	35
	GO:0042379	Chemokine receptor binding	1.20E-03	29
	GO:0051881	Regulation of mitochondrial membrane potential	1.40E-03	41
ATO+AMN107	GO:0019886	Antigen processing and presentation of exogenous peptide antigen via MHC class Ⅱ	8.00E-05	45
	GO:0002495	Antigen processing and presentation of peptide antigen via MHC class Ⅱ	1.10E-04	45
	GO:0008285	Negative regulation of cell proliferation	2.40E-04	29
	GO:0009069	Serine family amino acid metabolic process	5.20E-04	40
	GO:0031646	Positive regulation of neurological system process	5.30E-04	45
	GO:0050806	Positive regulation of synaptic transmission	5.30E-04	45
	GO:0051971	Positive regulation of transmission of nerve impulse	5.30E-04	45
	GO:0097458	Neuron part	6.90E-04	45
	GO:0030424	Axon	9.10E-04	30
	GO:0030903	Notochord development	9.60E-04	29

ID: identity

In AMN107 group, time-series genes significantly associated with negative regulation of cellular carbohydrate metabolic process and chemokine receptor binding revealed an increasing-stable-increasing-decreasing (profile 29) expression trend. The time-series genes significantly correlated with peptidase activity, acting on L-amino acid peptides, chloride channel complex, and peptidase activity functions showed an increasing-stable-increasing-decreasing expression trend (profile 39; table 4).

In ATO+AMN107 group, the time-series genes which were associated with antigen processing and presentation, and neural signal transmission showed an increasing-decreasing-stable expression trend (profile 45). The time-series genes enriched in negative regulation of cell proliferation presented a stable-increasing-stable expression trend (profile 29; table 4).

Notably, in ATO group, seven time-series genes (*CCNA2*, *DAB2*, *EMP2*, *PODXL*, *PRSS2*, *PTK2B*, and *PTMA*) that were significantly enriched in cell adhesion showed an increasing-decreasing expression trend (profile 40). In ATO+AMN107 group, five time-series genes (*AP2M1*, *CLTC*, *DNM2*, *FCER1G*, and *SEC24B*) that were significantly associated with antigen processing and presentation of peptide antigen via MHC class Ⅱ presented an increasing-decreasing-stable expression pattern (profile 45; table 5).

Table 5 The expression patterns of time-series genes that were significantly related with the top ten GO items

Drug treatment	Category ID & Name	Gene symbol	Gene number	GO adjusted *P*-value	Profile ID	Profile *P* -value
ATO	GO:0045785 Positive regulation of cell adhesion	CCNA2, DAB2, EMP2, PODXL, PRSS2, PTK2B, PTMA	7	0.006	40	1.0E-07
ATO+AMN107	GO:0019886 antigen processing and presentation of exogenous peptide antigen via MHC class Ⅱ	AP2M1, CLTC, DNM2, FCER1G, SEC24B	5	0.034	45	1.0E-04

ID: identity

3 DISCUSSION

In this study, different time-series genes having different expression patterns were identified by treatment with ATO, AMN107, and the combination of the two drugs, respectively. Among these genes, those in the ATO+AMN107 group had noticeably different expression patterns compared to time-series genes in the other two groups in profile 29 and 49. In addition, expression change trends in profile 40 were different between the ATO group and combination group. The enrichment analysis showed that time-series genes with significant expression patterns had different functions in different treatment groups. In the ATO group, time-series genes (e.g. *CCNA2* and *DAB2*) that were associated with cell adhesion functions presented increasing-decreasing trend (profile 40), while in the combination group, time-series genes which were significantly associated with serine family amino acid metabolic process presented increasing-stable-increasing trend (profile 40). In the AMN107 group, time-series genes with expression pattern similar to profile 29 (stable-increasing-decreasing) revealed significant association with cellular carbohydrate metabolic process, whereas in the ATO+AMN107 group, time-series genes in profile 29 (stable-increasing-stable) were pronouncedly associated with negative regulation of cell proliferation. In addition, time-series genes (e.g. *AP2M1*) mainly enriched in antigen processing-related functions were found to present an increasing-decreasing-stable expression trend (profile 45).

The different expression patterns for time-series genes between the ATO (or AMN107) group and ATO+AMN107 group could be explained by the different gene functions. Cell to cell adhesion signaling is implicated in CML, and several genes, such as *CTNNA1* and *ACTA1*, in this pathway are upregulated in CML hematopoietic stem cells[18]. CyclinA2 (*CCNA2*) is a cell cycle-related gene that mainly functions in the G_1/S and G_2/M phases[19]. In CML, the expression of CCNA2 protein is downregulated by imatinib administration alone or in combination with arsenic sulfide[20]. The cell cycle-related genes also have function during cell adhesion. For instance, the cell cycle-related genes such as *COL6A1* and *API3* are reported to promote cell adhesion[21]. However, cell adhesion can activate cell cycle progression[22]. Cell cycle and cell adhesion are regulated by the same enzyme 2-O-sulfotransferase[23]. These observations collectively suggest the close relationship of the two cellular activities. Notably, ATO compels the CML-initiating cells (LICs) to enter the cell cycle by downregulating PML expression. This process enhances the drug sensitivity of CML cells[24]. These findings reveal that ATO administration might eradicate the TKI resistance of CML cells through regulation of cell cycle-related genes.

The inhibition of forkhead O transcription factors (FOXO) is critical for the proliferation of CML cells, and this process results from the activation of Akt signaling triggered by BCR-ABL[25]. Moreover, it has been shown that TGF-β-FOXO pathway plays a vital role in the maintenance of CML LICs[25]. The clathrin adaptor protein (DAB2, also known as Disabled-2) is an adhesion-responsive phosphoprotein, which plays an important role in cell adhesion[26]. Notably, *DAB2* is reported to modulate TGF-β signaling in cancer cells[27]. Taken together, DAB2 might also exert its function in CML LICs following ATO drug treatment via involvement of cell adhesion.

In our present study, the two genes *CCNA2* and *DAB2*, which were specific time-series genes in the ATO group, had different expression patterns (in profile 40, increasing-decreasing) compared with the other two groups, suggesting the two genes might exert their functions immediately after the ATO administration (due to the increased expression), while the functions might be attenuated with time (due to the decreased expression). Importantly, they were significantly enriched in the positive regulation of cell adhesion function, suggesting that ATO might influence the expression of genes involved in cell cycle or cell adhesion, thereby eliminating TKI resistance in CML cells.

The time-series genes in AMN107 group were closely related to energy metabolism (cellular carbohydrate metabolic process), and these genes showed a similar expression pattern as profile 29 (stable-increasing-decreasing), indicating the time-series genes after AMN107 administration might not work at first, and after a little duration, they might exert their functions. Likewise, their functions might be weakened over time. Cellular carbohydrate metabolic process is essential in tumor cells owing to vigorous proliferation[28]. AMN107 is a novel aminopyrimidine inhibitor of BCR-ABL in both imatinib-sensitive and imatinib-resistant CML. In particular, AMN107 shows potent activity against imatinib-resistant CML owing to quantitative alterations to the BCR-ABL protein in the activation site[29]. During this process, extensive cellular carbohydrate metabolisms might be involved, and it is understandable that following AMN107 treatment, the altered genes were significantly enriched in this metabolic process. Based on previous findings and the results of enrichment analysis, it can be inferred that AMN107 might influence energy metabolism, and the genes involved in this process might be used as targets for imatinib-resistant CML therapy.

The combination of ATO and AMN107 demonstrated a synergistic effect on CML treatment[9]. In addition, based on our present enrichment analysis, we observed that the time-series genes in the ATO+AMN107 group were markedly different from those in ATO or AMN107 group, suggesting that ATO+AMN107 therapy might have different effects on gene expression and their functions. Compared to the AMN107 group, time-series genes in the ATO+AMN107 group, which had expression pattern similar to profile 29 (stable-increasing-stable), were highly correlated with the regulation of cell proliferation. The expression trend suggests that following ATO+AMN107 treatment, the time-series genes might need a short time before their response to these drugs. Notably, after response to the drugs, they might function throughout the remaining time duration. Aberration of several genes is reported to impact cell proliferation. In K562 CML cell lines, overexpressed *CCN3* decreases proliferation and enhances sensitivity to imatinib[30]. Moreover, increased *IFNα* expression could directly inhibit cell proliferation in CML cells[31]. Additionally, compared to the ATO group, time-series genes that had expression pattern in profile 40 (increasing-stable-increasing) were significantly related to serine family amino acid metabolic process. The expression pattern indicated that time-series genes having this trend might have an immediate response to the combination treatment, and then might be inactive. Function of these genes might be recovered after a while. Adaptor related protein complex 2 Mu 1 subunit (*AP2M1*) belongs to the family of adaptor complexes medium subunits. Reportedly, it is involved in antigen processing in the MHC class Ⅱ and Ⅰ pathways in human monocytes[32]. Monocytes could derive dendritic cells (DCs) from the blood of CML patients[33]. Notably, imatinib could impair the generation of matured DCs from normal monocytes[34]. This provides a hint that the combination therapy of ATO+AMN107 might also block DC formation similar to imatinib. All these suggested genes related to antigen processing might also be altered after ATO+AMN107 treatment. Collectively, ATO+AMN107 therapy might cause alterations in genes involved in the cell proliferation process, serine family amino acid metabolism, and antigen processing in the MHC class Ⅱ and Ⅰ pathways in imatinib-resistant CML cells.

Although we performed a comprehensive time-series analysis, this study has certain limitations. First, the sample size was relatively small because only one sample was present in each group at each time point. Second, the intervals between the sampling time points were not equidistant, and this might cause errors in the expression pattern graph generated by the STEM software. Third, the gene expression experiments were not validated.

In conclusion, the time-series genes in imatinib-resistant CML cell lines under the three treatments have different biological functions. ATO treatment might influence cell cycle- and cell adhesion-related genes, AMN107 might affect genes involved in cellular carbohydrate metabolism, while the combination therapy might regulate genes involved in cell proliferation and antigen processing function. However, further experimental validations are needed to confirm our results.

Conflict of Interest Statement

The authors declare that there is no conflict of interest with any financial organization or corporation or individual that can inappropriately influence this work.

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