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Overexpression of CHK1 and CHK2 in pediatric patients of B-acute lymphoblastic leukemia

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Abstract

Background: Despite breakthroughs in the development of chemotherapy drugs to treat pediatric B-acute lymphoblastic leukemia (B-ALL), the relapse rate remains a major therapeutic challenge, requiring more detailed characterization of molecular elements underlying disease development and resistance to treatment. Checkpoint kinase 1 (CHK1) and checkpoint kinase 2 (CHK2) are two critical mediators of the DNA damage response (DDR) mechanism that activate the downstream components responsible for DNA repair, cell cycle regulation, and apoptosis. It has been shown that altered expression of CHK1 and CHK2 in various tumor entities promotes tumorigenesis and disease progression.

Materials and Methods: In this case-control study, we evaluated the relative expression status of CHK1 and CHK2 genes in pediatric B-ALL patients at diagnosis (n=20), during complete remission (n=23) and relapse phase (n=10), as well as 20 peripheral blood samples from healthy children as a normal control group. The mRNA expression levels of CHK1 and CHK2 were determined by the Real-time PCR method. Data were compared using the Mann–Whitney U test for the relative expression level of target mRNA in different phases of B-ALL. Data were presented as median and statistical significance was described as a P-value less than 0.05.

Results: Our results revealed that CHK1 expression increased in newly diagnosed patients than in healthy individuals ($p \leq 0.001$). Relapsed patients had higher CHK1 expression than the newly diagnosed ($p \leq 0.05$) and complete remission ($p \leq 0.001$) counterparts. CHK2 was overexpressed in all phases of the diseases ($p \leq 0.001$) without any significant alteration among the studied groups.

Conclusion: Given the CHK1 ability to endow cancer cells with a survival advantage upon chemotherapy, the present study suggests it as a potentially promising target in the fight against B-ALL.

Keywords: Acute lymphoblastic leukemia, Checkpoint kinase 1, Checkpoint kinase 2

Introduction

B-acute lymphoblastic leukemia (B-ALL) is considered the most common cancer diagnosed in children, responding well to therapy in 85-90% of cases (1). Although chemotherapy as the first-line and standard treatment has enhanced pediatric patients' overall survival with ALL, some subsets are refractory to induction therapy (1, 2). Therefore, countless studies have emphasized the investigation of molecular mechanisms responsible for ALL development to increase treatment efficiency. Chemotherapy drugs are very effective in targeting highly proliferative cancer cells by inducing DNA damage; however, malignant cells can take advantage of a multifaceted signaling pathway called DNA damage response (DDR) to withstand the lethal effects of genotoxic agents (3). Mammalian cells use DDR machinery, consisting of a group of DNA repair pathways and cell-cycle checkpoint processes to maintain genome

stability (4). In the presence of peculiar DNA structures produced upon DNA damage, cell cycle progression is stoped by cell cycle checkpoints to allow time for DNA repair mechanisms to remove DNA injuries; though, irreparable damages trigger apoptosis (5). The initial responder to DNA injuries is the Phosphatidylinositol-3 kinase-like protein kinases (PIKK) family, including ataxia telangiectasia mutated (ATM) as well as ATM and Rad3 related (ATR). ATM and ATR are activated by DNA damages and cell cycle progression inhibition, and authorization of repair are mediated via ATR and ATM-dependent phosphorylation of CHK1 (checkpoint kinase 1) and CHK2 (checkpoint kinase 2), respectively (6). Both CHK1 and CHK2 phosphorylate Cdc25 phosphatases eventually prevent entry into mitosis and S-phase (7, 8).

Upregulated CHK1 and CHK2 expression upon treatment with cytotoxic drugs induce cell cycle block and DNA repair commencement that confers cancer cells resistance to chemotherapy drugs (9). Overexpression of CHK1 has been verified as a common incident in numerous cancer types including colon, gastric, breast, nasopharyngeal and hepatocellular carcinoma, etc. (10-15). Interestingly, CHK1 expression frequently correlates with tumor grade and chemoresistance (12, 14). Therefore, this can elucidate why checkpoint kinases inhibitors are promising agents for combined-modality strategies to bypass refractory to chemotherapy (16-19). Nonetheless, few studies highlighted the role of CHK1 and CHK2 gene expression alteration in acute lymphoblastic leukemia patients. In the present study, we have analyzed the mRNA expression level of CHK1 and CHK2 in different clinical phases of pediatric B-ALL. Consistent with the oncogenic function of CHK1 and CHK2, our results indicated a high expression level of CHK1 in newly diagnosed and relapsed cases in relation to complete remission or healthy individuals, suggesting a potential correlation between CHK1 and B-ALL etiology.

Materials and Methods

Patients

During the study, a total of 53 peripheral blood samples were collected from the various clinic stages of pediatric B-ALL; there were 20 newly diagnosed patients, 23 complete remissions (day 33 of induction chemotherapy) samples and ten relapsed cases from Rasul Akram Hospital and Mahak Hospital and Rehabilitation Complex (Tehran, Iran). The details of the patients are givenin Table I. Moreover, as controls, peripheral blood samples of 20 age-matched healthy children who did not have any signs of disease were taken from Masoud Clinical Laboratory (Tehran, Iran). According to WHO criteria (based on immunophenotyping, cytogenetic analysis and cytomorphology), diagnosis of B-ALL was made mainly. All newly diagnosed B-ALL patients received the same treatment according to the Berlin-Frankfurt-Munster (BFM) protocol. The presence of less than 5% blasts in the bone marrow, absence of leukemic blasts in peripheral blood and CSF, and no evidence of extramedullary disease was considered as complete remission and relapse was defined as the recurrence of ≥ 25% lymphoblasts in BM and/or localized leukemic infiltrates at any site.

RNA isolation and cDNA synthesis

Mononuclear cells were separated from the peripheral blood (PBMCs) through Ficoll density gradient centrifugation (PAN BIOTECH, Germany) and washed 2 times with 1X phosphate-buffered saline. Then, the cell pellet was lysed using 1 ml TRIzol reagent (Life Technologies, USA) according to the manufacturer's instructions. 1% agarose gel electrophoresis for the identification of

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18S and 28S rRNA bands, as well as spectrophotometer NanoDrop (Thermo Scientific, USA) for assessing A260/A280 nm ratio and A260/A230 nm ratio were used for evaluating RNA quality and quantity respectively. Contaminating genomic DNA was digested with DNase-I (Thermo Scientific, USA), and 1 µg of total RNA was reverse transcripted into cDNA using Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). The quality of synthesized cDNA was verified by regular PCR and running on the agarose gel (1.5%) using β-actin.

qRT-PCR

Reactions of qPCR were performed on Light Cycler 96 Real-time PCR system (Roche Diagnostics, Lewes, UK) using RealQ Plus 2x Master Mix Green (Ampliqon, Denmark). According to the manufacturer's protocol, the total volume of 15 µl was prepared for each reaction containing 1.5 µl amplified cDNA, 7.5 µl SYBR Green MasterMix, 4 µl distilled water, 1 µl of 10 µmol of each forward and reverse primers. Primer sequences of genes, which were designed exon/exon junction to inhibit genomic DNA amplification, are shown in Table 2. The examination for each sample was carried out in duplicate. Amplification conditions were initial activation at 95 °C (initial denaturation step) for 15 min following 40 cycles of 15s (denaturation step) at 95°C and 60 s at 60°C (Annihilig step). Relative expression of the CHK1 and CHK2 gene were investigated by the comparative C_t method ($2^{-\Delta\Delta Ct}$) (20), using the β-actin reference gene as a normalization factor and endogenous control. Furthermore, the specificity of the PCR products was evaluated by melting curve analysis.

Statistical analysis

Normality of the variables distribution was evaluated by the Kolmogorov-Smirnov test. Due to nonparametric distributions, data were compared using the Mann–Whitney U test for the relative expression level of target mRNA in different phases of B-ALL. All above statistical test were accomplished by the SPSS statistical software, version 25. Data were presented as mean and statistical significance was described as a P-value less than 0.05.

Ethical Consideration

This study was permitted by the Medical Ethics Committee of Iran University of Medical Sciences (agreement number IR.IUMS.REC.1397.32663).

Results

Expression levels of CHK1 in pediatric B-ALL patients at diagnosis, during complete remission and relapse.

As presented in Figure 1a, the relative expression level of CHK1 was increased significantly in all phases of B-ALL, including newly diagnosed (n=20), complete remission (n=23), and relapsed cases (n=10) compared with healthy donors (n=20) (fold chang: 21.14, $p \leq 0.001$; fold chang: 9.35, $p \leq 0.001$ and fold chang: 22.63, $p \leq 0.001$ respectively). Although CHK1 expression was decreased in patients achieving complete remission compared with the new case group, the alteration was not statistically meaningful ($p \geq 0.05$). CHK1 was highly overexpressed in relapsed cases related to both the new case ($p \leq 0.05$) and the complete remission group ($p \leq 0.001$).

Expression levels of CHK2 in pediatric B-ALL patients at diagnosis, during complete remission and relapse.

As indicated in Figure 1b, there was a meaningful increase in the relative expression level of CHK2 in all stages of B-ALL, including newly diagnosed (n=20), complete remission (n=23), and relapsed cases (n=10) in comparison with healthy individuals (n=20) (fold chang: 6.61, $p \leq 0.001$; fold chang: 5.58, $p \leq$

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0.001 and fold chang: 17.49, p ≤ 0.001 respectively). No remarkable alterations regarding CHK2 expression were detected among new cases, complete remission, and relapsed patients ($p \geq 0.05$), and a steady-state of CHK2 expression was observed at different clinical phases of the disease.

Table I: Demographic characteristics and laboratory findings of patients

Characteristics	New diagnosis case (n= 20)		Complete remission cases (n=23)		Relapse cases (n=10)	
Age	≤ One year	2	≤ One year	2	≤ One years	0
	1-10 years	11	1-10 years	13	1-10 years	6
	≥ 10 years	7	≥ 10 years	8	≥ 10 years	4
Gender	Male	15	Male	13	Male	9
	Female	5	Female	10	Female	1
WBC count	< 50,000 (/µl)	11	< 50,000 (/µl)	15	< 50,000 (/µl)	4
	≥ 50,000 (/µl)	9	≥ 50,000 (/µl)	8	≥ 50,000 (/µl)	6

Table II: The sequence of primers used for quantitative real-time PCR

Gene	Primers	Sequence	Tm	Size (bp)
CHK1	Forward	5´-ATATGAAGCGTGCCGTAGACTG-3´	60.54	**154**
	Reverse	5´-AGCTCTCCTCCACTACAGTACTC-3´	60.37	
CHK2	Forward	5´-TGATGTCTCGGGAGTCGGATG-3´	61.36	**120**
	Reverse	5´-TGGATATGCCCTGGGACTGTG-3´	61.60	
Actin	Forward	5´-GGAAATCGTGCGTGACATTAAG-3´	58.33	**181**
	Reverse	5´-GAAGGAAGGCTGGAAGAGTG-3´	57.89	

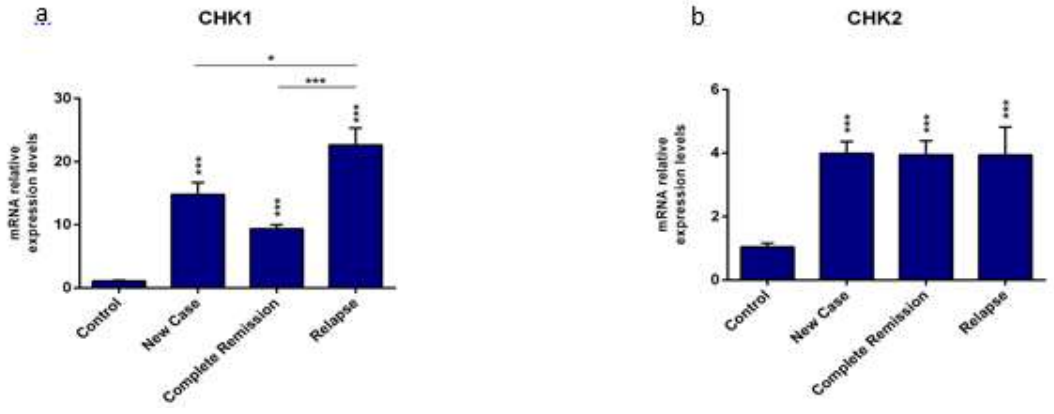

Figure 1. Expression level of CHK1 (a), and CHK2 (b) genes in pediatric B-ALL patients at diagnosis, during complete remission, and relapse. The results are expressed as mean with SEM. *P ≤ 0.05, ***P ≤ 0.001 compared to control group.

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Discussion

Approximately 85% of pediatric B-ALL treated with current chemotherapeutic regimens attain remission. However, disease recurrence remained a hurdle that hampers successful treatment in some ALL patients (21, 22). Cytotoxic agents create DNA damage and induce cell death, but cancer cells can employ DDR pathways to survive (23). Once DNA damage occurs, CHK1 and CHK2 are phosphorylated by ATR and ATM, respectively, resulting in the activation of downstream proteins responsible for DNA repair, cell cycle regulation, and apoptosis (24). It has been reported that the altered expression of CHK1 and CHK2 in various tumor entities contributed to the cancer risk. Furthermore, tumor cells may obtain chemotherapy resistance through augmented checkpoint kinases expression (11-15, 25).

It has been suggested that CHK1 exerts both anti- and pro-tumoral function depending on cellular context or in response to various oncogenic signals (9, 26). In our investigation, CHK1 expression status was higher in newly diagnosed and relapsed B-ALL patients in relation to healthy control and complete remission groups (Fig. 1a). Consistent with our findings, an elevated CHK1 transcript level was reported in T-ALL patients at diagnosis compared to normal thymocytes. The results specified CHK1 role in protecting malignant cells against enhanced replication stress following unrestrained cell cycle progression through inhibition of cell death induced by ATM/caspase-3 (17). Moreover, higher mRNA expression levels of CHK1 but not CHK2 have been observed in 8 B/T ALL cell lines and 54 adults newly diagnosed ALL patients in comparison with normal bone marrow mononuclear cells (27). Di Rorà AGL research team also reported that CHK1, but not CHK2, is significantly over-expressed in blasts of ALL patients in comparison to normal mononuclear cells (28). Similarly, David et al. (29) reported enhanced CHK1 expression in AML patients and suggested that it was an independent prognostic marker in AML. Likewise, CHK1 was overexpressed in both primary and imatinib-resistant leukemia cells of CML cases (30). Altogether, previous studies corroborate our findings for proposing a tumor-promoting role for CHK1 in pre-B ALL cells. Aberrant CHK1 expression in B cell-progenitor ALL cells mitigate DNA damage stresses resulted from chemotherapeutic drugs or replicative stress and eventually, this can lead to tumor recurrence (10). CHK1 protein is also involved in the regulation of homologous recombination (HR) DNA repair pathway. When DNA damage occures, CHK1 phosphorylates the RAD51 protein, one of the key components of the HR pathway, which stimulates the recruitment of RAD51 to the site of damage (31, 32). Thus, monitoring the expression of this gene could be a valuable prognostic marker. Moreover, based on the CHK1 function in guarding leukemia cells against chemotherapeutics-induced DNA damages, CHK1 inhibitors, as chemosensitizers, can improve the cytotoxicity of these drugs (17).

The investigation of mRNA and protein expression of CHK2 in various cancers has not been well studied. In a study by Tort et al. (33) similar expression levels of CHK2 protein and mRNA in all types of lymphomas were shown. However, no significant difference in CHK2 expression levels in NHLs was found. We revealed that CHK2 mRNA generally displayed higher expression in pediatric B-ALL patients compared with the healthy control group, but it did not differ according to the stages of the disease (Fig. 1b). Our finding is in line with Iacobucci et al. (34) study

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reporting that mRNA and protein expression of CHK2 was increased in 68% of primary ALL blast cells of adult patients. However, it has been shown that the CHK2 gene expression is downregulated in Hodgkin's lymphoma cells via epigenetic mechanisms (35). Likewise, downregulation of CHK2 expression due to the methylation of gene promoter has been reported in non-small cell lung cancer (NSCLC) cell lines and clinical specimens of Lung cancer which involved in chemoresistance of NSCLC (36).

It must be acknowledged that this study also had some limitations. As discussed above, the patients in this study were all treated with BFM chemotherapy regimen, whereas different standard protocols may be used in different treatment centers, an aspect that may influence the expression levels of CHK1 and CHK2 genes. Apart from that, for this study, peripheral blood samples were used to investigate the expression of the desired genes; if the results could also be performed on bone marrow samples of the patients, more favorable results would be obtained. Last but not least, because the cytogenetic analysis data of a number of patients were not available, it was not possible to evaluate the correlation between cytogenetic changes and the expression level of the above genes.

Conclusion

Overally, we reported a relatively high expression of CHK1 during the proliferative stages of pediatric ALL patients. Likewise, CHK2 expression was higher in BCP-ALL cases than the healthy control group. A plausible hypothesis is that concomitant increase of CHK1 and CHK2 expression levels in pediatric BCP-ALL patients may mediate the disease's progression through up-regulation of the DNA repair genes. For instance, the interaction between CHK1-RAD51 has an essential role in protecting malignant cells against the lethal effects of DNA lesions (37). Also, this mRNA expression pattern may be beneficial in designing novel therapeutic strategies for the treatment of leukemia. Given the small sample size of this patient population, further study of checkpoint kinases expression in a larger group of patients appears warranted. Moreover, additional investigation of gene expression alterations in DDR-related genes may enrich our knowledge of B-ALL pathogenesis and facilitate the recognition of cases at increased risk of this disease.

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Conflict of interest

The authors declare no conflict of interest.

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