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ORIGINAL ARTICLE

Triangle collaboration assessment of autophagy, ER stress and hypoxia in leukemogenesis: a bright perspective on the molecular recognition of B-ALL

Fatemeh Feizi[a], Mehdi Allahbakhshian Farsani[b], Amin Mirzaeian[b], Vahide Takhviji[a], Abbas Hajifathali[b] and Mohammad Hossein Mohammadi[b]

[a]Laboratory Hematology and Blood Banking Department, School of Allied Medical Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran; [b]HSCT Research Center, Laboratory Hematology and Blood Banking Department, School of Allied Medical Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran

ABSTRACT

B-lineage acute lymphoblastic leukemia (B-ALL) is the most common acute leukemia in childhood and adults, which caused by many various crystalline and unclear agents. Owning to this matter, no significant progress has been made in the patients-recovery. Recently, autophagy pathway is considered as an ambiguous agent in leukemia evaluation. We aim to discover the expression levels of upstream autophagy-regulating genes in newly diagnosed B-ALL patients. In B-ALL group, *BECN1*, *HIF1A* and *ERN1* expressions were significantly down-regulated, while *BCL2* expression was up-regulated compared to the control group ($p < .05$). Moreover, there was significant positive correlation between the decreased *BECN1* compared with Hypoxia and endoplasmic reticulum (ER) stress-related genes expression in the patients ($p < .05$). Our findings revealed that, *ERN1* and ER stress pathway-related genes could be effective regulators of autophagy in B-ALL. More investigation is recommended to gain a deeper understanding into molecular pathophysiology of B-ALL to improve treatment and monitoring approaches in affected patients.

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Introduction

Upon the improvement in knowledge of the deregulated cellular functions in leukemia, autophagy has been noticed, dramatically (Hassen *et al.* 2017). Basal macroautophagy (hereafter referred to as autophagy), a multi-step recycling process, is responsible for cellular homeostasis maintenance under changing or stress conditions such as starvation, misfolded protein accumulations, endoplasmic reticulum (ER) stress, strictly like a "intracellular wall" (Jin *et al.* 2018). Though in the malignancy, depend on the type of tumour, stage of disease and autophagy up-stream activator/inhibitor signalling pathways, this mechanism could play dual roles as tumour suppressor and promoter (Helgason *et al.* 2011).

Due to up/down-regulation of autophagy in malign cells, transcriptional regulation of autophagy-related genes could be especially unique and substantial in every cancer type (Bolliet *et al.* 2017). In this regard, large changes in the expression levels of autophagy-related genes have been documented in the several malignancies, particularly in leukemia (Yue *et al.* 2003). The first robust association between autophagy and cancer was proved after the discovery of *BECN1* as a haploin sufficient tumour suppressor. *BECN1*, one of the core components of the autophagy machinery, is part of phosphatidylinositol 3-kinase complex that initiates the formation of the autophagosome isolation membrane in nucleation step (Miracco *et al.* 2007). Decreased expression of the *BECN1* encoding gene has been correlated with tumour progression in many diverse solid tumours including ovarian, breast and brain cancers, with a lower survival rate in the affected individuals (Yue *et al.* 2003). Nevertheless, state of the *BECN1* regulation pattern at transcriptional level has been a point of controversy in leukemia (Radwan *et al.* 2016).

B-cell acute lymphoblastic leukemia (B-ALL), a heterogeneous hematologic neoplasm, accounts for approximately 80% of ALL cases along with numerous various courses, which its heterogeneity provokes researcher in exploring autophagic trajectories in ALL (Farsani *et al.* 2017).

Molecular research has been illustrated how an interaction between the *BECN1* and up-stream pathways is able to regulate autophagy. As mentioned above, *BECN1*-dependent autophagy might be tightly regulated by its binding partners, regulator signalling pathways and related transcriptional factors (Helgason *et al.* 2011). In this respect, a number of papers have been shown that hypoxia inducible factor 1 subunit alpha (*HIF1A*) as a hypoxic transcriptional factor could induce autophagy by up-regulation of BCL2 interacting protein 3 (*BNIP3*) and *BNIP3* Ligand (Füllgrabe *et al.* 2016). The hypoxia-inducible gene *BNIP3*, as a constitutive component of autophagy, is also the proapoptotic

CONTACT Mohammad Hossein Mohammadi drmohammadi@sbmu.ac.ir HSCT Research Center, Laboratory Hematology and Blood Banking Department, School of Allied Medical Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran

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member of the B-cell lymphoma-2 (*BCL2*) family that could contribute to *BECN1*-activation through inhibition of the *BCL2*-activity (Murai *et al*. 2005b). Indeed, *BCL2* is a pivotal molecule in the autophagy and apoptosis interaction along with down-regulation performance of the both of them by interacting with the BH3 domain of *BECN1* (Nencioni *et al*. 2013). Furthermore, under ER stress, ER to nucleus signalling 1 triggers *BCL2*-phosphorylation through c-Jun N-terminal kinase 1 (*JNK1*)/*JUN* pathway and consequently dissociation of *BECN1* from *BCL2*. Accordingly, proautophagic activity of *BECN1* could be increased following *BCL2* suppression (Füllgrabe *et al*. 2016). In another pathway, death-associated protein kinase 1 (*DAPK1*), a tumour suppressor and cell suicide promoter gives rise to *BECN1* phosphorylation at the BH3 domain, this in turn induces autophagy (Kang *et al*. 2011).

Given discrepancies among previous studies, we aimed to examine the expression level of *BECN1, BCL2, BNIP3, HIF1A, JNK1, ERN1* and *DAPK1* in Iranian B-ALL patients compared with healthy individuals to better comprehension of impressive autophagy regulator pathways in B-ALL. Hence, the precise identification of these pathways will be a gleam of hope diagnostic purposes, patients monitoring and more helpful ALL-classification in future.

Materials and methods

Study population

From June 2017 to August 2018, Bone Marrow (BM)-samples obtained from 50 untreated patients with B-ALL, none of whom had been treated previously and 20 healthy controls, with informed consent. The Consent letter was approved by the research ethics committee at the Shahid Beheshti University of Medical Sciences. The patient samples were diagnosed BMT laboratory, Taleghani hospital, according to morphology characteristics, defining specific immunophenotypes and molecular analysis. BM samples were extracted from individuals who invited for physical examination and have no hematologic malignancies and/or obvious abnormalities in their blood tests.

RNA extraction and cDNA synthesis

Total cellular RNA was extracted and purified from mononuclear cells using RNeasy Kit (Qiagen, Germany) according to the manufacturer's protocol. The quality and quantity of extracted RNA was evaluated by NanoDrop (Thermo Scientific, Wilmington, North Carolina, USA) (optical density 260/280 nm ratio >1.8). Subsequently, 2 μL (0.5 mg) RNA was utilised for cDNA synthesise in a final volume of 20 μL using a Thermo Scientific kit (Qiagen, Hudson, NH, USA). c-DNA synthesis was checked by ABL primer as housekeeping gene.

Real-time quantitative PCR

Specific primers were designed for target genes (*BECN1, BCL2, BNIP3,* HIF-1α, *JNK1, ERN1* and *DAPK1*) and ABL as an internal control gene using GeneRunner software (details were shown in Supplementary Table A1). The mRNA expression level of selected genes was measured via real-time quantitative polymerase chain reaction (qRT-PCR) (Rotor-Gene 6000, Qiagen, Germany). The qPCR components for each reaction was composed of 2 μL of template target cDNA, 1 μL of forward and reverse primer, 7.5 μL of Real Plus 2x Master Mix Green-Low ROX (Ampliqon, Denmark) and 4. 5 μL water to reach total volume of 15 μL. Primer efficiency was estimated from a standard curve using four consecutive 1:10 dilutions of cDNA sample (1, 0.1, 0.01 and 0.001) for each target gene. All experiments were performed in triplicate and the relative quantification of mRNA expression for each sample (fold change = FQ) was calculated via the Livak method (2-ΔΔCT) (Livak and Schmittgen 2001).

Statistical analysis

All the statistical analyses were performed using the SPSS software for windows (version 16.0) and the GraphPad Prism 6 software. Gene expression levels were calculated according to the 2-ΔΔCt formula, where ΔΔCt = (Ct target gene − Ct ABL) sample−(Ct target gene − Ct ABL) calibrator. Shapiro-Wilk and the Kolmogorov-Smirnov tests were utilised to evaluate the normal distribution of target genes expression in ALL patients and the control group. Regarding the state of normal distribution, Student *t* or Mann–Whitney *U* tests were used. Finally, to study the possible correlation between variables parametric distribution, Pearson's correlation test was employed. p values of less than .05 were considered statistically significant.

Results

Characteristics of ALL patients at the time of sample collection

The demographic and laboratory (or clinical) information of 50 patients with *de novo* ALL were shown in Table A2.

BECN1, BCL2, BNIP3, HIF1A, JNK1, ERN1 *and* DAPK1 genes expression in ALL patients and healthy subjects

According to the obtained finding, the significant *BECN1, HIF1A* and, *ERN1* down-regulation and *BCL2* up-regulation have been observed in ALL patients in compared with normal control group. Additionally, the expression levels of *BNIP3, JNK1* and *DAPK1* was decreased but it was not significant ($p > .05$) (Figure A1).

Correlation between BECN1, BCL2, BNIP3, HIF1A, JNK1 *and* ERN1 *expression levels in studied groups*

Statistical correlation analysis indicated that a significant positive correlation exists between gene expression levels of *BECN1* and *HIF1A* ($r = 0.33$, $p < .01$), *BECN1* and *BNIP3* ($r = 0.30$, $p < .05$), *BNIP3* and *BCL2* ($r = 0.37$, $p < .01$), *BECN1* and *ERN1* ($r = 0.35$, $p < .01$), *ERN1* and *JNK1* ($r = 0.44$,

$p < .0001$), *JNK1* and *BCL2* ($r = 0.41$, $p < .01$) and strong positive correlation between *HIF1A* and *BNIP3* ($r = 0.66$, $p < .01$) in ALL patients. The *BECN1* and *BCL2* expression levels showed a positive correlation but it was not significant ($p > .05$) (Figure A2).

Discussion

Despite dramatic therapeutic advances in recent years, overall survival for patients still remains roughly 5-year (Terwilliger and Abdul-Hay 2017). So, comprehensive inquiry of intra- and extracellular ALL-developing agents is seriously compulsory for improvement of therapeutic strategies (Khosravi *et al.* 2018). Due to the importance of the autophagy machinery in malignancy development and progression (Nencioni *et al.* 2013), we decided to shed light on the autophagy transcriptional complexities, as a pivotal facet of autophagy regulation in ALL.

As the expression and prognostic significance of *BECN1* as a key component of autophagy in ALL are largely unexplored (Miracco *et al.* 2007), we selected this gene for study. First, we evaluated the expression levels of *BECN1* up-stream genes in the *ERN1*-dependent (ER stress) signalling pathways in patients with B-ALL in comparison to healthy subjects. According to this pathway, the expression of *ERN1*, *JNK1* and *BCL2* genes were analysed. Our findings have been denoted significant *ERN1* down-regulation and *BCL2* up-regulation in ALL patients. Also, reduced expression of *JNK1* mRNA was observed in 40 percentages of the patients. Hereafter, we assumed that *BECN1* expression might be affected. In the next step, transcriptional level analysis of *BECN1* was shown that *BECN1* mRNA expression was lower especially in the patients.

In order to explain our findings, a recent study conducted by Abdelsalam *et al.* exhibited *BCL2* over-expression and *BECN1* under-expression in *de novo* ALL patients compared with remission group (Abdelsalam *et al.* 2017). Another investigation represented down-regulation of *BECN1* and the negative correlation between *BECN1* and *BCL2* in hepatocellular carcinoma (Qiu *et al.* 2014). We could point to various mechanisms for proving the tumour suppressive role of *BECN1,* including angiogenesis inhibition, mutant frequency reduction and cell cycle retardation (Radwan *et al.* 2016). Besides, it seems that incremental regulation of *BCL2* as an oncoprotein in different types of human cancers is along with accelerated growth of tumour, metastasis and poor prognostic. Indeed, *BCL2* promotes crosstalk between apoptosis and autophagy and with own mutual features could be benefit for malignant cell maintenance and progression (Um 2016). In contrast with our outcomes, *BECN1* over-expression and autophagy hyperactivity was observed in a number of studies. For instance, in Yi-Lin Kong's investigation, higher expression levels of *BECN1* mRNA and protein were detected in patients with chronic lymphoblastic leukemia (CLL) vs. control group. Due to an intensive resistance of leukemic lymphocyte to apoptosis, here increase of autophagy as a cell death strategy might be an alternative compensatory mechanism for apoptosis (Kong *et al.* 2018). Since, autophagy induction is essential modulator of leukemic Long Term-Hematopoietic Stem Cells maintenance in the BM osteoplastic niche, the expression of autophagy-specific genes in chronic myeloid leukemia Imatinib-resistant patients was higher than the healthy population (Rothe *et al.* 2014). Above mentioned findings led us to note here that depending on cancer cell types and under diverse contexts, autophagy could bring cells to the different destiny.

Concerning JNK and *ERN1*, Bujisic *et al.* exhibited that the gene expression of IRE1 was blocked by epigenetic suppressors in GCB-DLBCL cancer samples. Substantially, IRE1 in another pathway engages own endoribonuclease agency in activation of XBP1, which in turn drive B-cell differentiation toward plasma cell (Bujisic *et al.* 2017). In this manner, *ERN1* down-regulation could contribute to both of lymphoblast maturation arrest and blockade of apoptosis in the ALL. Inconsistent with our results, in solid tumour, hyperactivity of IRE1is responsible for angiogenesis and tumour development through induction of VEGF-A expression (Auf *et al.* 2010). With respect to *JNK1,* Cellurale *et al.* elucidated that the *JNK* loss-of-function mutations could increase the chance of breast cancer formation, since *JNK1* takes part in genome stability protection (Cellurale *et al.* 2012). Inconsistency, Jian Cui's finding illustrated that hyperactivation of *JNK1* might lead leukemic cells to stepped-up cell cycle promotion and resistance to Fas-mediated apoptosis in T-ALL (Cui *et al.* 2009). Taken together, based on the *JNK1* or *ERN1* upstream/downstream signalling molecules, one could imagine paradoxical functions for both of them in malignancies.

Now, one question is whether *BECN1* is only under the influence of ER-stress or further pathways? Hence, we evaluated two other principal paths: cell death and hypoxia.

For cell death signalling, *DAPK1* mRNA was analysed and it was decreased in 76 percentages of the patients. This finding was confirmed by many publications; Sarhan *et al.* exhibited the *DAPK1* promoter hypermethylation in acute myeloid leukemia (AML), ALL and CLL groups compared with the control subjects (Sarhan *et al.* 2016). Other researches demonstrated that *DAPK1* function and expression might be decline in several solid tumors including breast, ovarian and liver cancer and so on (Li *et al.* 2017). Consequently, *DAPK1* down-regulation could be defensible, owning to its crucial role in controlling the cell cycle, programed cell death and metastasis repression (Singh *et al.* 2016, Nikbakht *et al.* 2017).

Then, for Hypoxia pathway, the levels of *HIF1A* and *BNIP3* genes expression were evaluated. The *BNIP3* under-expression was observed in 58% of the patients. To consolidate the above result, Murai *et al.* detected the methylation and silencing of *BNIP3* in a number of ALL, AML and multiple myeloma samples (Murai *et al.* 2005a). In contrast, high *BNIP3* expression in several solid tumours is associated with extensive invasiveness and poor survival (Macher-Goeppinger *et al.* 2017). Actually, these heterogeneous outcomes stem from contradictory *BNIP3*-roles as immune evasion stimulator in solid tumours or hypoxia cell death inducer in hematologic malignancies (Murai *et al.* 2005a). Elevated expression of HIFs was reported in several cancers, which could mimic

as oncogenes in order to enhance of cell proliferation, angiogenesis and tumour progression (Carroll and Ashcroft 2006). Nonetheless, in our study *HIF1A* down-regulation was detected. To resolve these discrepancies, one could point to two scenarios: First, some evidence has proved that *HIF1A* can act as a tumour suppressor gene. For instance, in murine AML, *HIF1A* loss-of-function mutant can lead to accelerated leukemogenesis by undirected activation of certain oncogenes (Velasco-Hernandez *et al.* 2014). Second, HIF2A encoding gene has high homology in sequence and functions with *HIF1A,* so it might be responsible for proliferation, angiogenesis and glucose metabolism upon prolonged hypoxic conditions (Dengler *et al.* 2014). Consequently, *HIF1A* down-regulation could possess prosurvival feature via autophagy/apoptosis inhibition.

Conclusion

In conclusion, in accordance with our findings, down-regulation of ER-stress pathway could noticeably give rise to decreased expression of *BECN1* and subsequently autophagy in B-ALL samples. Albeit, hypoxia alongside Programmed cell death can do regulation of *BECN1*-dependent autophagy but *ERN1* is more vigorous, here. Maybe the decreased expression level of IRE1 could provoke both of leukemic blasts prosurvival and differentiation suspension, together. Prospectively, IRE1 and its associated genes would be considered as hallmark and impressive biomarkers in newly risk stratification, prognosis determination and monitoring in B-ALL.

Disclosure statement

There is no conflict of interest among authors.

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