

ORIGINAL PAPER

HEAT SHOCK PROTEINS 70 AS MARKERS OF COMPLICATIONS IN CHILDREN WITH ACUTE LYMPHOBLASTIC LEUKEMIA

Tetiana KONDRATIUK¹✉, Yuriy ODINETS¹, Tetiana GORBACH², Victoria KOVAL¹

¹ The 2nd Department of Pediatrics, Kharkiv National Medical University, Kharkiv, Ukraine

² Department of Biological Chemistry, Kharkiv National Medical University, Kharkiv, Ukraine

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ABSTRACT

Introduction. The mortality rate from complications of acute lymphoblastic leukemia (ALL) in children is high and exceeds the mortality rate from relapse of leukaemia. Ultrahigh expression of HSP 70 is associated with treatment resistance and is an adverse clinical outcome in ALL in children.

The objective of the study was to evaluate the dynamics of the level of HSP 70 in the blood serum of children with ALL.

Material and methods. 46 children with ALL were studied. Before the start of treatment, the level of HSP 70 in the blood serum has been studied (n = 46). In case of appearance of complications during the conduction of the remission induction, the HSP 70 has been restudied in blood serum (n = 29).

Results. HSP 70 is significantly increased in children with ALL. A significant decrease in HSP 70 in the blood serum of patients with induction therapy was revealed compared with the initial level of this protein (p < 0.05). No correlation between the level of HSP 70 in the blood serum of children with ALL and the manifestations of chemotherapy complications has been found. In contrast to previous studies, we

RÉSUMÉ

Les protéines de choc thermique 70 en tant que marqueurs des complications de la leucémie aiguë lymphoblastique chez l'enfant

Introduction. Le taux de mortalité par complications de la leucémie aiguë lymphoblastique (LAL) chez les enfants et son traitement reste élevé et dépasse le taux de mortalité par récurrence de leucémie. La surexpression des protéines de choc thermique 70 est associée à une résistance thérapeutique et à des résultats cliniques défavorables dans la leucémie aiguë lymphoblastique chez les enfants.

L'objectif de l'étude. Nous avons étudié la dynamique des niveaux de protéine de choc thermique 70 dans le sérum des enfants atteints de leucémie aiguë lymphoblastique.

Matériel et méthodes. 46 enfants atteints de leucémie aiguë lymphoblastique ont été examinés. Avant le traitement, les enfants ont été évalués pour la protéine de choc thermique 70 dans le sérum (n = 46). Pendant les complications pendant l'induction de la rémission, une nouvelle détermination des protéines de choc thermique 70 dans le sérum a été effectuée (n = 29).

✉ Address for correspondence:

Tetiana KONDRATIUK
The 2nd Department of Pediatrics, Kharkiv National Medical University,
Kharkiv, Ukraine
Address: 4 Nauky Avenue, 61022, Kharkiv, Ukraine
Email: kmnutetiana@gmail.com; Phone +380978584772

did not find a significant effect of HSP 70 on mortality.

Conclusions. Induction therapy leads to a decrease in the level of HSP 70 in the blood serum of children with ALL. We did not find a direct correlation between the occurrence of complications during induction therapy and HSP 70 concentration in the blood serum.

Keywords: heat shock proteins 70, complications, acute lymphoblastic leukemia.

Abbreviations:

HSP 70 – heat shock proteins 70

HSP – heat shock proteins

ALL – acute lymphoblastic leukemia.

ALL IC BFM 2009 – Acute Lymphoblastic Leukaemia Intensive Chemotherapy Berlin Frankfurt Munich 2009

Me – median

Lq – lower quartile

Uq – upper quartile

ROC – receiver operating characteristic

ELISA – Enzyme-Like Immunosorbent Assay

AUC – area under curve

Se – sensitivity

Sp – specificity

INTRODUCTION

In Ukraine, the acute leukemia in children has a prevalence of 4.6:100,000 children, with a leukemia mortality rate of 1.0:100000¹. The prognosis of the disease is unfavorable, as a result of the disease progression itself and chemotherapy, in addition to infectious-toxic complications. Nowadays, despite the success in treatment, the side effects and toxicity of modern protocols remain significant problems of hematology²⁻⁴.

In 2018, a group of scientists led by Ana Paula Trussardi Fayh⁵, in a study of 19 patients with acute lymphoblastic leukemia (ALL), proved that heat shock proteins 72 (HSP 72) are involved not only in blast transformation and leukemia progression, but also have immunological and metabolic functions. Thus, they can be used as markers of metabolic, inflammatory and oxidative changes in ALL in children during chemotherapy⁵.

The history of the study of high-conservative HSP⁶ in various conditions and onco-hematology dates back to the discovery of HSP by the scientist Ritossa F. in 1962⁷. These proteins are the first to respond to changes in the internal environment (homeostasis) and trigger protective adaptive reactions. Nowadays, 6 HSP families are known, including HSP 70, which are the most studied.

Résultats. Les protéines de choc thermique 70 sont significativement augmentées chez les enfants atteints de leucémie aiguë lymphoblastique. Des réductions significatives des protéines de choc thermique 70 dans le sérum des patients pendant le traitement d'induction ont été trouvées par rapport à la valeur initiale de cette protéine ($p < 0,05$). Aucune relation n'a été trouvée entre les niveaux de protéine de choc thermique 70 dans le sérum des enfants atteints de leucémie aiguë lymphoblastique et les manifestations des complications de la chimiothérapie. Contrairement aux études précédentes, nous n'avons trouvé aucun effet significatif de la protéine de choc thermique 70 sur la mortalité.

Conclusions. La thérapie d'induction entraîne une diminution du taux de choc thermique 70 dans le sérum sanguin des enfants atteints de LAL. Nous n'avons pas pu trouver de corrélation directe entre la survenue de complications pendant le traitement d'induction et la concentration de choc thermique 70 dans le sérum sanguin.

Mots-clés: protéines de choc thermique 70, complications, leucémie aiguë lymphoblastique

Ultra-high expression of HSP, including HSP 70, is associated with treatment resistance and an adverse clinical outcome in ALL in children⁶, due to the fact that blast cells have their own HSP 70, with a cytoprotective effect. As a result, HSP 70 tumors work against the patient, protecting pathological cells from destruction and contribute to the development of blast cells' resistance to chemotherapy⁸. To date, according to existing studies, HSP 70 has only a negative role in the development and progression of leukemia. However, it is worth noting that most of the researches have studied the level of heat shock proteins in blast cell structures. The number of studies of HSP 70 in the blood serum is much smaller, especially in regard of the pediatric patients with ALL. In Ukraine, such studies have not been conducted.

THE OBJECTIVE OF THE STUDY was to assess the dynamics of the level of HSP 70 in the blood serum of children with ALL.

MATERIALS AND METHODS

The study was performed between September 1st, 2016 and November 1st, 2018. 46 children (31 boys and 15 girls) with ALL who were hospitalized at the Kharkiv City Clinical Children's Hospital N°

16, Ukraine, were studied. The patients' age ranged from 1 to 17 years. The control group consisted of 19 healthy children of the same age and gender presented to Kharkiv City Outpatient Hospital № 16 for routine health control or vaccination.

The diagnosis of ALL was established according to the diagnostic criteria specified in the Acute Lymphoblastic Leukaemia Intensive Chemotherapy Berlin Frankfurt Munich 2009 (ALL IC BFM, 2009)⁹. After the diagnosis has been established, the patients began an inductive chemotherapy course (I protocol) under the program ALL IC BFM 2009⁹.

The criteria for inclusion in the study were verified diagnosis of ALL, signed consent from parents and/or patients. The criteria for exclusion in the study were refusal of the parents or/and patients to sign the consent or death of patients.

Heat shock proteins 70

Before the initiation of treatment (leukemia manifestation), the level of HSP 70 in the blood serum has been studied in children (group A, n = 46). If complications occurred during remission induction, the HSP 70 has been restudied in blood serum (group B; n = 29). The HSP 70 has been studied in children from the control group once, during a routine examination by a pediatrician.

The serum HSP 70 assessment has been performed by enzyme immunoassay using a kit of reagents for the quantitative determination of human HSP 70 "Heat shock proteins 70 high sensitivity ELISA kit" by Enzo Life Sciences (Switzerland) according to the available instructions. The results of HSP 70 have been evaluated in ng/mL.

Treatment

According to the protocol ALL IC BFM 2009⁹, induction of remission for both at-risk groups included the following drugs administration: prednisolone 60 mg/m²/d (days 1-28), vincristine 1.5 mg/m²/d (days 8, 15, 22, 29), daunorubicin 30 mg/m²/d (days 8, 15, 22, 22), L-asparaginase 5000 IU/m²/d (days 12, 15, 18, 21, 24, 27, 30, 33), mercaptopurine 60 mg/m²/d (days 36-63), cyclophosphamide 1000 mg/m²/d (days 36, 64), mesna 1:1 cyclophosphamide, cytarabine 75 mg/m²/d (days 38-41, 45-48, 52-55, 59-62), intrathecal therapy – methotrexate (days 1, 12, 33, 45, 59). In addition to chemotherapy, all patients received accompanying therapy, which was at the discretion of the attending physician, according to the recommendations of the ALL IC BFM 2009 protocol⁹.

Complications

Complications were clinical, laboratory and documented deterioration of the patient's condition

during chemotherapy. Hematologic complications were defined as: neutropenia (absolute neutrophils quantity in the blood <1500 /mkl) and "visible" bleeding. Complications of the gastrointestinal tract manifested in the form of: toxic hepatitis (with an increase in alanine-aminotransferase level to 60 IU/L, which is higher than the upper normal value 40 IU/L) in the blood serum and toxic gastro-enteropathy (abdominal pain, nausea and vomiting, food refusal, repeated liquid stool). Infectious complications included: stomatitis (mucosal defects with white bloom, pain, and food refusal), acute bronchitis (hyperthermia and cough without physical or radiological signs of pneumonia), and pneumonia (hyperthermia, intoxication syndrome, cough, physical and radiological signs). Organ dysfunction or multi-organ failure was recorded in the case of a rapid deterioration in hemodynamics, rapid breathing, decreased oxygen blood composition, absence of urine output, and the need for artificial lung ventilation and /or hemodialysis. There were not any other types of chemotherapy complications during the study.

Ethics approval and consent to participate

Each study participant and his /her parents were informed about the nature of the study and signed a consent to participate in the study. The study was approved by the Ethics and Bioethics Committee of Kharkiv National Medical University, Ukraine (Protocol No. 8 of 5th October 2016) and was conducted according to Helsinki Declaration (1975).

Statistical analyses

For statistical analyses of data STATISTICA 8 (Tulsa, OK) has been used. Shapiro-Vilka test has been used for verification of the distribution according to the Gauss law. Non-parametric variables included median (Me), interquartile range [Lq – lower quartile; Uq – upper quartile]. To compare the two dependent samples, the non-parametric Wilcoxon test (T) was used. To compare two independent samples, non-parametric Mann-Whitney U-test has been used. All P-values were two-tailed, and values <0.05 have been considered significant. ROC analysis was used to evaluate the specificity and sensitivity of the method.

RESULTS

The study involved 46 children with ALL. General characteristics of patients are highlighted in Table 1. There was a significant prevalence of boys than girls (67.4% and 32.6%, p = 0.025). Patients had a median age of 5.2±4.5 years (range 1 to 17 years). All the patients were Caucasians. Among the

Table 1. General characteristics of patients at diagnosis.

Characteristics	n = 46	%
Gender male / female	31 / 15	67.4 / 32.6
Age, years		
< 3	15	32.6
3-6	21	45.7
7-10	4	8.7
11-14	1	2.2
15-18	5	10.8
Race White / Other	46 / 0	100 / 0
Immunophenotype B - lineage / T - lineage	36 / 10	78.3 / 21.7
Risk group standard / high	33 / 13	71.7 / 28.3

Table 2. Frequency of complications of acute lymphoblastic leukaemia in patients of group B

Complications	Total		I phase n = 29	II phase n = 29	
	n = 29	%			
Hematologic	Neutropenia	22	75.8	12	10
	Bleeding	5	17.2	4	1
	Aphthous stomatitis	8	27.5	5	3
Infectious	Acute bronchitis	4	13.7	4	0
	Pneumonia	4	13.7	1	3
Complications of the gastrointestinal tract	Toxic hepatitis	21	72.4	13	6
	Toxic gastro-enteropathy	9	31.0	6	3
	Respiratory failure	2	6.8	1	1
Insufficient organ / system function	Acute renal failure (on the background of lysis syndrome)	1	3.4	1	0
	Cardiovascular failure	1	3.4	0	1
	Multiple organ dysfunction syndrome	1	3.4	0	1

immunophenotypic leukemia variants, B cell lineage leukemia prevailed ($p = 0.008$). A larger number of children under study (71.7%) had a standard risk group ($p = 0.006$).

Among the 46 children, the material was collected successfully from only 29 in case of complications during induction therapy (group B). The nature of the complications that were recorded and the period of their diagnosis (up to 34 days of treatment - phase I, 34-64 days - phase II) are presented in Table 2. The patients from group B simultaneously had a combination of complications, which is associated with the mechanism of action of the above-mentioned chemotherapeutic drugs. As indicated in Table 2, neutropenia occurred in 75.8% of patients with protocol phases I and II. 72.4% of children had manifestations of toxic hepatitis during both phases of induction therapy. 31.0% of the patients developed toxic gastro-enteropathy, which was also recorded throughout the protocol I. In most cases, infectious processes

(aphthous stomatitis, acute bronchitis, pneumonia) were associated with neutropenia, so they occurred during phases I and II. Complications of individual organ/organ systems dysfunction in the studied patients were recorded in isolated cases.

HSP 70 level in blood serum of patients of group A (before treatment) was 5.51 ng/mL (3.69-8.93 ng/mL). The patients from group B had a serum level of HSP 70 of 4.79 ng/mL (2.89-7.45 ng/mL). The concentration of HSP 70 in the blood serum was 0.45 ng/mL (0.38-0.68 ng/mL) (Figure 1) in children from the control group, who at the time of the study had no signs of acute or chronic diseases' manifestations. There was a significant difference in serum HSP 70 levels between patients of group A, B and control: Mann-Whitney U-test with A group-control group $p=0.000$; Mann-Whitney U-test with group B - control group $p=0.000$ (Figure 1).

After the initiation of the therapy, even with the addition of complications, a significant decrease

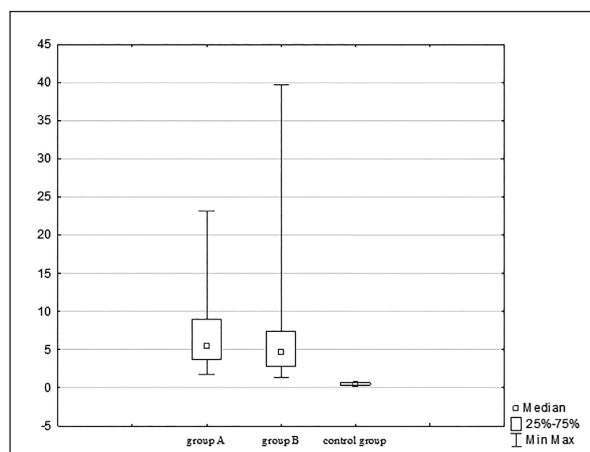


Figure 1. The level of HSP 70 (ng/mL) of children in groups A, B and control

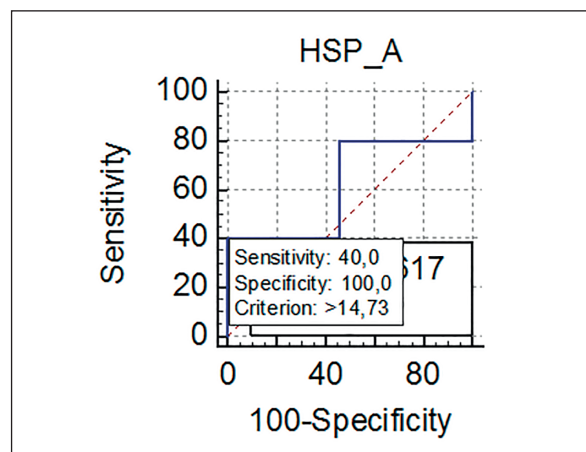


Figure 2. ROC prediction curve of the fatal outcome likelihood from HSP 70 during the leukemia.

Table 3. ROC indicators of heat shock proteins 70 level and complications of induction therapy for acute lymphoblastic leukemia

Parameter	AUC	"cut-off point"	Se, %	95% CI	Sp, %	95% CI	+LR	-LR
Hematologic complications	0.591	>7.45	28.6	11.3-52.2	100.0	54.1-100.0		0.71
Complications of the gastrointestinal tract	0.591	<7.45	86.4	65.1-97.1	60.0	14.7-94.7	2.16	0.23
Infectious complications	0.591	<7.45	92.9	66.1-99.8	38.5	13.9-68.4	1.51	0.19

in the level of HSP 70 was recorded compared to its initial level: Wilcoxon test (T) group A-B $p = 0.0129$.

We did not find a statistically significant correlation ($p > 0.05$) between the clinical characteristics of chemotherapy complications and the level of HSP 70 in blood serum at the time of the deterioration of the child's health status. ROC - the analysis demonstrates the lack of ability to predict the onset of three main groups of complications (hematologic, infectious, and gastrointestinal complications) that occurred in our patients, according to HSP 70 in blood serum (Table 3).

10.8% (5/46) of patients had a fatal evolution. Among 5 deaths, 3 children (6.5%) died from complications of therapy during the Induction Protocol under the ALL IC BFM 2009 program. Two children (4.3%) died after the end of our study, due to recurrence of ALL. Analyzing the level of HSP 70 among patients who died, according to the ROC - analysis (Figure 2), there was no significant effect of the initial level of HSP 70 in the blood serum on the occurrence of a fatal outcome in our cohort of patients with ALL ($p > 0.05$).

DISCUSSION

Acute leukaemia is an oncological disease that is more commonly present in childhood in patients

with ALL and in male patients^{2,10,11}. There was a significant ($p = 0.025$) prevalence of boys (67.4%) than girls (32.6%), which is consistent with the literature data on the significant proportion of ALL among boys¹⁰.

The onco-hematologic process is an absolute stress for the children's organism. That is why, in the course of leukaemia, there is an increase in the expression of stress proteins - HSP 70⁶. In our study, the expression of HSP 70 in children with ALL is significantly higher than the expression of HSP 70 in healthy patients, which is consistent with the literature data¹² and is a response to the oncological process in the patient's body¹³.

A significant decrease in HSP 70 in the blood serum of patients with induction therapy was revealed compared with the initial level of these proteins ($p < 0.05$). Our results coincide with the results obtained in the study of 2018⁵, where the authors also found a decrease in the level of HSP 72 in the blood serum of children with ALL after induction therapy. Such dynamics of heat shock proteins have been explained by the fact that induction therapy leads to a decrease in the activity of the oncological process, improvement of the patient's health status and normalization of metabolic and oxidative reactions. Based on the fact that HSP 70 combines several heat shock proteins

that have a molecular weight between 66 and 78 kD¹⁴, we assume that a decrease in HSP 70 due to induction chemotherapy is also a favorable sign, which reflects a decrease in oncological load.

Despite the gradual decrease in HSP 70 during the treatment protocol I, it remains significantly higher than the same level in healthy children. This allows us to assume that HSP 70 continues to have a separate, unknown part in modulating the future course of leukemia.

In the course of the study, we did not find a correlation between the level of HSP 70 in the blood serum of children with ALL and the manifestations of chemotherapy complications. According to ROC analysis, HSP 70 concentration does not predict the occurrence of complications in a single organ/ organ system. This may be due to the fact that HSP 70 are not specific, they are markers of ontogeny^{13,15}, other pathological conditions^{16,17} and respond to any changes in homeostasis^{18,19}. In order to determine the final role of these proteins during the various stages of leukemia, further studies are needed, involving more patients.

In contrast to previous studies^{5,12}, we found no significant impact of HSP 70 level on mortality. We explain these discrepancies by the small number of deaths in our study and the difference between patient samples.

Today, the mortality rate from complications of ALL in children and its therapy remains high and exceeds the mortality rate from relapse of leukaemia^{2,20}. These data are the same as ours: 3 (6.5%) children out of 46 died from complications of the underlying disease, complications of therapy, or a combination of these two pathological conditions. Only 2 children (4.3%) died as a result of relapse of leukaemia. Moreover, the mortality period for patients with complications of polychemotherapy fell on protocol I, which coincides with the literature data on the high probability of mortality during this initial stage of treatment³.

Limitations of the study:

1. We were unable to study the level of HSP 70 in dynamics in a whole cohort of patients, because of refusal of parents.
2. We faced difficulties in predicting the presence or absence of individual complications, depending on the level of HSP 70 in the blood serum, due to the fact that chemotherapy disrupted several organ systems at once.
3. In our sample, there was not a large number of fatal cases that could affect the uncertainty of forecasting according to ROC analysis.

CONCLUSIONS

HSP 70 is significantly increased in children with ALL. Induction therapy leads to a decrease in the level of HSP 70 in the blood serum of children with ALL. However, at the same time, it does not normalize and remains significantly higher than the indicators of the control group.

We were unable to find a direct correlation between the occurrence of complications during induction therapy and HSP 70 concentration in the blood serum.

Due to a high mortality rate secondary to complications during the treatment of acute lymphoblastic leukemia in children, the issue of the scientific research of early prognostic markers of chemotherapy complications remains a challenge.

Author contributions

Conceptualization: T.K. and Y.O.; Methodology: T.K. and Y.O.; Software: T.K. and V.K.; Investigation: T.G.; Formal analysis: T.K.; Statistic analysis: T.K. and V.K. Writing – original draft preparation: T.K., Y.O., and V.K.; Writing – review and editing: Y.O. and T.G.; Visualization: T.K.; Supervision: Y.O. and T.G. All the authors have read and agreed with the final version of the article.

Compliance with Ethics Requirements:

„The authors declare no conflict of interest regarding this article“

„The authors declare that all the procedures and experiments of this study respect the ethical standards in the Helsinki Declaration of 1975, as revised in 2008(5), as well as the national law. Informed consent was obtained from all the patients included in the study“

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