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Mitogen-Activated Protein Kinase 2 Relevance for the in-vitro Chemo-Sensitivity and Clinical Response in Children with Acute Lymphoblastic Leukemia in Mansoura University Hospitals

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Mitogen-Activated Protein Kinase 2 Relevance for the in-vitro Chemo-Sensitivity and Clinical Response in Children with Acute Lymphoblastic Leukemia in Mansoura University Hospitals

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ABSTRACT

Background: Identification of different prognostic biomarker of pediatric acute lymphoblastic leukemia (ALL) permits more characterization of patient subgroups and helps the risk-adapted treatment strategies. **Aim:** Our study assessed the role of Mitogen-Activated Protein Kinase (ERK1/2) as a prognostic biomarker of pediatric ALL in MUH patients. Another aim is to detect the relevance of ERK1/2 for the in-vitro leukemic cells response to chemotherapeutic agents (Vincristine, L-asparaginase Dexamethasone and Doxorubicin). **Material and Methods:** ERK1/2 was measured in the bone marrow aspirates of 26 children with newly diagnosed ALL by ELISA technique. The MTT in vitro chemo-sensitivity assay was used to detect in-vitro leukemic cells response. **Results:** At diagnosis, the pERK 1/2 and tERK 1/2 are significantly higher in the HR gp than SR gp (p-value =0.000 & 0.003 respectively). While the pERK/tERK 1/2 ratio at diagnosis was significantly higher than that post induction in the patients of our study (p-value= 0.03). A significant association was detected between in vitro chemo-sensitivity to vincristine, l-asparaginase, dexamethasone & doxorubicin and complete remission after induction therapy, respectively). **Conclusion:** Our results indicated that ERK1/2 could be considered as an independent predictor of complete remission. Besides, a significant positive correlation was detected between ERK 1/2 and in vitro leukemic cells response to the four chemotherapeutic agents at diagnosis.

Keywords: acute lymphoblastic leukemia, ALL, MAPK, ERK, TERK, Chemosensitivity

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INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common pediatric malignancy worldwide and represents about 30% of all childhood cancers (Inaba and Pui, 2021). Although now in high-income countries the 5-year cancer survival exceeds 85%, these advances poorly translate to low- and middle-income countries. In a retrospective cohort study of children with cancer (2007 – 2017) at the Children's Cancer Hospital Egypt (CCH), the most common childhood cancer reported was ALL. In that retrospective study, the 5 - year survival for ALL over the two diagnosis periods (2007-2012 & 2013-2017) was approximately 78% (Soliman et al., 2021).

Risk stratification of patients with childhood leukemia depends on factors that are considered when decisions are made about the intensity of chemotherapy. The major prognostic factors include age, initial WBC count at diagnosis, immunophenotype, cytogenetic features, and early response to treatment (Hunger and Mullighan 2015). The identification of different biologic prognostic factors permits the characterization of patient subgroups with distinct relapse risks and the realization of risk-adapted treatment strategies (Brown et al., 2020).

The Mitogen-Activated Protein Kinase (MAPK) pathway is one of the most important mechanisms of eukaryotic cell regulation. It adjusts vital cellular processes such as growth,

proliferation, differentiation and apoptosis by controlling the transcription of many genes (Son et al., 2011). The MAPK pathway consists of a set of three successively acting kinases: a MAPK kinase kinase, a MAPK kinase, and a MAPK. The MAPK is also called ERK (extracellular-signal-regulated kinases). Once classic ERKs, ERK1 & ERK2 (ERK1/2), are activated, they phosphorylate different substrates in the cytosol and nucleus to bring the appropriate biological response (Cargnello and Roux 2011).

Abnormal regulation of MAPK pathway has been linked to carcinogenesis. Mutations that cause deteriorations in the components of MAPK pathway are among the roots of leukemia pathogenesis (Al-Kzayer et al., 2015). Mutations in the Ras gene and consequently in Ras proteins are highly prevalent in leukemia. The gain-of-function mutations cause the GTP that interacts with Ras, to stay permanently attached to Ras. As a consequence of this, the Ras pathway remains open and leads to continuous activation of ERK1/2 and accordingly the uncontrolled proliferation signals in the lymphoblast (Akin et al., 2021). In a study by Kim and co-workers (1999), the activation of ERK was tested in about 80 human acute leukemia samples by in vitro kinase assay and immune-blot analysis. Their results suggested that ERK and MAPK kinase were constitutively activated in acute leukemia (Kim et al., 1999).

The chemotherapy resistant leukemic cells exhibited a significantly higher expression of MAPK cascade components. Furthermore, inhibition of MAPK kinase (1 & 2 subtypes) enhanced the glucocorticoid-induced cell death. There was an increased in vitro dexamethasone activity in ALL blasts from approximately 90 % of tested patients as a result of MAPK kinase suppression (Polak et al., 2016).

The execution of in vitro chemo-sensitivity tests as a predictive tool in clinical practice is among the most vital future aims in oncology. The drug resistance/sensitivity can be determined accurately by in vitro chemo-sensitivity assays. This was revealed in a number of studies of leukemia and solid tumors. Moreover, the

results of these assays can help prediction of outcome (Popova and Levkin, 2020).

The Methyl Thiazolyl Tetrazolium (MTT) in vitro chemo-sensitivity assay on cultured leukemic cells had been reported to have high sensitivity (about 90 %) and high specificity (about 80 %), as well false negative result was not detected (Dogan et al., 2004; Jun et al., 2007). Using in vitro chemo-sensitivity assay test in arrangement with information from molecular profiling can afford a fuller picture about the nature of individual disease. Moreover, the most essential goal is to correlate in vitro response with clinical outcomes for each particular chemotherapeutic agent. This possibly facilitates the individualization of patient treatment plans (Jain, 2021).

PATIENTS AND METHODS

Patients

The present study was carried out on twenty-six patients with newly diagnosed ALL (with no previous treatment). The patients were selected from pediatric outpatient clinic at Mansoura oncology center in the period from July 2018 to July 2019. Informed consent from all parents of patients was given to their participation in this study. The study was approved by the Research Ethics Committee (REC) for experimental & clinical studies and IRB (MD/17.11.81) in Mansoura University/ Egypt. Patients were diagnosed by history taking, clinical examination and standard investigations including complete blood count, blood film and bone marrow aspirate for cytogenetics and immuno-phenotyping studies.

Immunophenotyping

Immunophenotyping evaluation has been done using the following mixture of monoclonal antibodies (MoAbs) panels for acute leukemia diagnosis and to identify lineage affiliation, stage of maturation, and further characterization of acute leukemia: Cells were stained with fluorescein-conjugated Sm mAb against CD19, CD20, CD10, CD2, CD5, CD7, CD4, CD8, CD13, CD33, CD117, CD 64, CD14, CD36, CD33, CD34, and HLA-DR. Intracellular mAb as cytoplasmic CD 79a, cyto CD3, MPO, and TdT. Samples on a BD FACS Canto™ flow cytometer was analyzed. All monoclonal antibodies were

purchased from BD Pharmingen, San Diego, CA, USA. Acquisition and study of flow cytometric data was conducted by the FACS DIVA software (BD, USA).

The patients were divided in two groups according to the risk group stratification was based on Children's Oncology Group (COG) system criteria. Thirteen patients of our study were considered standard risk group (SR gp) with initial WBCs count was $< 50 \times 10^3/\mu\text{l}$ at diagnosis and age ranged from one year to ten years and no T-ALL and no other high risk (HR) criteria. The other thirteen patients had two or more HR criteria and considered high risk group (HR gp).

Procedure

EDTA- anti-coagulated bone marrow (BM) aspirate samples from 26 patients were taken for routine diagnosis & post induction of remission therapy (after 4 weeks). These samples were used for isolation and culturing of mononuclear cells, and also for Mitogen-Activated Protein Kinase (ERK1/2) protein detection in the cell lysate.

Isolation of mononuclear cells (MCs) from BM samples (Riedhammer et al., 2014)

All steps were done in a laminar flow cabinet. Laminar flow cabinet has a UV-C germicidal lamp to sterilize the interior and contents 30 minutes before usage to prevent contamination of experiment.

The sample was diluted with an equal volume of RPMI 1640 culture medium (Sigma-Aldrich). The diluted sample was carefully layered on top of a volume of Ficoll–Paque (Pharmacia, Uppsala, Sweden) equal to the original volume of the sample. The mononuclear cells were separated by density gradient centrifugation at 400 g for 20 min at room temperature. After centrifugation, sterile glass pipette was used to aspirate the PBMCs, and cells were transferred to the 15-ml tubes. RPMI 1640 culture medium was added to PBMCs then centrifugation was done at 500 g for 10 min followed by aspiration of the supernatant. RPMI 1640 culture medium was added to MCs pellet with gentle mix.

The cell viability was determined using trypan blue dye exclusion method and counting the viable cells by hemocytometer under light

microscope (Strober, 2015; Zwaan et al., 2000). The PBMCs were cryopreserved. Culture medium (RPMI 1640 90 %v + fetal bovine serum 10%v) were added to the PBMCs. An equal volume of freezing medium (fetal bovine serum 80%v + DMSO 20%v) was added in three subsequent steps in intervals of 3–4 min. PBMCs suspensions were transferred to the cryovials and freeze in -80 C freezer as fast as possible then to liquid nitrogen (store them until use)

Thawing and resting of Mononuclear cells (MCs) (Riedhammer et al., 2014)

Samples were placed on ice after removal from liquid nitrogen, and then they were thawed in a 37°C water bath. In 10 ml falcon tube contained warm RPMI-1640 medium, the cells were then transferred and centrifuged for 5 minutes at 500 g. This step was repeated and the cell pellet was re-suspended in medium. After that PBMCs were rested overnight to remove any apoptotic cells (the freshly thawed PBMCs incubated for approximately 18 hours). Finally, PBMCs were washed and ready to be used in culture or any other assay. The cell viability was determined using trypan blue dye exclusion and viable cells were counted by hemocytometer under light microscope¹⁶

In vitro chemo-sensitivity testing (Zwaan et al., 2000; Galderisi et al., 2009)

The cells were suspended in culture medium containing RPMI-1640 supplemented by 10% fetal calf serum penicillin (100 IU/ml) and streptomycin (100 g/ml). The concentrations of drugs in the medium were: vincristine 0.5 µg/ml, l-asparaginase 10 IU /ml, dexamethasone 1.4µg/ml and doxorubicin 0.5 µg/ml. In a 96-well culture plate, the cell suspension (180 µl) at a concentration 10^6 cells/ml was incubated with each drug solution (20µl). Control cells were cultured without drugs. The incubation last for three days humidified atmosphere (37°C & 5% CO₂)

Cell viability measurement by Methyl Thiazolyl Tetrazolium (MTT) (Mosmann, 1983)

- The mitochondrial dehydrogenases of living cells convert MTT (yellowish solution) to water- insoluble MTT formazan (dark blue crystals). The blue crystals are solubilized

and the intensity was measured colorimetrically at 570 nm.

- MTT was dissolved in phosphate buffered salt solution 5mg/ml.
- After culturing the cells (with & without drugs), MTT 20 μ l was added to each well.
- After incubation for four hours (the medium was discarded), the formazan crystals were dissolved with specific solubilization solution.
- Then at a 570 nm test wavelength, the absorbance was measured by plate reader.
- The optical density (OD) of both the incubated cells with each drug and the control cells were measured
- Leukemic Cell Survival Index % (LCSI %) was calculated as the following: $LCSI \% = (OD \text{ observed} / OD \text{ control}) \times 100\%$
- Less than fifty percent MTT viable cell was determined to be sensitive.
- More than fifty percent MTT viable cell was determined to be resistant.

Mitogen-Activated Protein Kinase (ERK1/2) protein detection by Enzyme Linked-Immuno-Sorbent Assay (ELISA)

Semi-quantitative detection of total Erk1&2 (regardless of phosphorylation state) and Erk1/2 double phosphorylated at threonine 185 and tyrosine 187 in cell lysates by sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA) (Sigma Aldrich).

Statistical analysis

With the aid of excel program and IBM SPSS program version, statistical analysis of data was done. Analysis of data was done via t- test (independent samples & paired samples) for comparison of variables inside and between groups ($P < 0.05$ was considered to be significant). Study of the correlation and association between the different variables either inside each group or between groups were performed using Pearson test, Spearman test & Chi square tests.

RESULTS

Patient characteristics

In this study we received bone marrow aspirates from 26 pediatric patients with newly diagnosed ALL (Table 1). The risk group stratification was based on Children's Oncology

Group (COG) system criteria (Hunger et al., 2013). Thirteen patients were considered standard risk group (SR gp) with initial WBCs count was $< 50 \times 10^3/\mu\text{l}$ at diagnosis (mean \pm SD = 24.12 ± 11.86) and age ranged from one year to ten years (mean \pm SD = 3.95 ± 1.77) and no T-ALL and no other high risk (HR) criteria. The other thirteen patients had two or more HR criteria and considered high risk group (HR gp) with WBC count (mean \pm SD = 80.64 ± 56.87) and the age (mean \pm SD = 8.68 ± 6.67).

Most of patients present with pallor, fatigue, fever, and bruising. Normocytic normochromic anemia was detected with hemoglobin was frequently less than 10 g/dl. Most of patients had thrombocytopenia. Seventeen patients had a history of recurrent infections and lymphadenopathy. Hepatosplenomegaly was demonstrated in six patients. Pleural effusion was detected in chest x-ray of two patients (had T-ALL).

At diagnosis, the pERK 1/2 and tERK 1/2 are significantly higher in the HR gp than SR gp (p -value = 0.000 & 0.003 respectively). The mean OD of pERK 1/2 was 0.55 ± 0.12 for HR gp versus 0.16 ± 0.03 for SR gp. While, the mean OD of tERK was 1 ± 0.18 for HR gp versus 0.67 ± 0.08 for SR gp (Figure 1A). After induction of remission chemotherapy phase, there were no significant differences of pERK and tERK 1/2 detected between the two gps. The mean OD of pERK 1/2 was 0.099 ± 0.022 for HR gp versus 0.092 ± 0.009 for SR gp (t -test p -value= 0.24). The mean OD of tERK 1/2 was 0.31 ± 0.04 for HR gp versus 0.29 ± 0.03 for SR gp (t -test p -value= 0.17) (Figure 1B). While, the pERK/ tERK 1/2 ratio at diagnosis was significantly higher than that post induction in the patients of our study (p -value= 0.03) (Figure 2).

A significant association was detected between pERK/ tERK 1/2 ratio (at diagnosis) and complete remission (CR) & prednisolone response (PR) in 26 children with ALL (Table 2). Complete remission was defined as no circulating blasts or extra-medullary, absolute neutrophil count $>1000/\text{l}$, platelets $>100,000/\text{l}$ and no recurrence for four weeks (Jasek et al., 2019).

Table 1. Characteristics of 26 children with newly diagnosed ALL

Characteristics	Number of Children
Sex	
Male	14
Female	12
Age (year)	
< 1	2
1-10	18
≥ 10	6
WBCs count	
< 50 x 10 ⁹ /l	19
≥ 50x10 ⁹ /l	7
Immunophenotyping (IPT)	
B -ALL	22
T-ALL	4
Cytogenetic studies	
Normal diploid	21
Hyper-diploid	3
Hypo-diploid	2
Events	
No complete remission	6

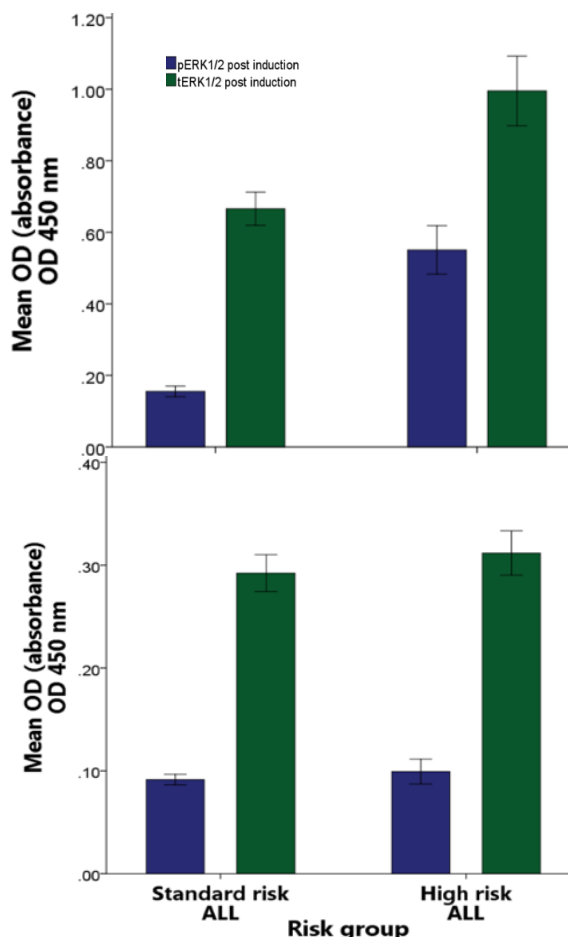


Figure 1. Difference of pERK & tERK in the standard risk (SR) and high risk (HR) ALL groups at diagnosis (A) & post induction therapy (B). (SR: standard risk ALL group, HR: high risk ALL group, OD: optical density)

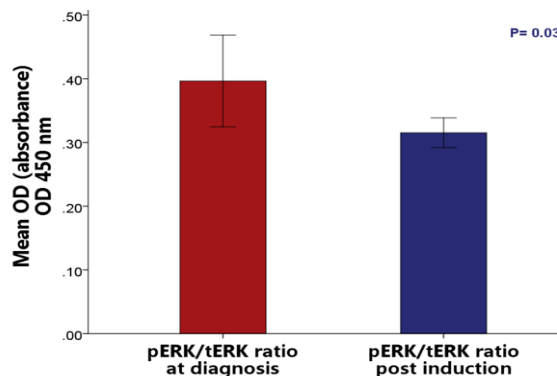


Figure 2. Difference of pERK/tERK 1/2 ratio at diagnosis & post induction therapy in all 26 patients with ALL.

Table 2. Association between pERK/ tERK ratio at diagnosis and complete remission (CR) & prednisolone response (PR) in 26 children with ALL

Parameter	pERK/ tERK 1/2 ratio ^b at diagnosis	t- test p- value
CR	No	0.609 ± 0.11
	Yes	0.332 ± 0.14
PR	Good	0.344 ± 0.15
	Poor	0.616 ± 0.11

Association is significant at < 0.05 level

Also, a highly significant correlation with moderate strength was found between pERK/ tERK 1/2 ratio and WBC count (Table 3).

Regarding in vitro chemo-sensitivity assay, the leukemic cells of diagnostic samples from the SR gp showed significantly higher in vitro sensitivity to vincristine, l-asparaginase and dexamethasone than the HR gp (P= 0.000, 0.001 and 0.000 respectively). As regard the in vitro response to doxorubicin, there was a little but significant difference between HR and SR gps. Both groups showed in vitro chemo-sensitivity to doxorubicin. The LCSi for doxorubicin was 37.61 (± 21.37) in HR gp, while in SR gp it was 20.39 (± 5.54) (p =0.01) (Table 4) (Figure 3).

Table 3. Correlation between pERK/ tERK 1/2 ratio and WBC count at diagnosis in 26 children with ALL

	Spearman correlation	pERK/tERK 1/2 ratio at diagnosis ^a
WBC count ^b	R	0.67
	P value	<0.001*
	Linear regression	
	Beta	0.53
	P value	0.005*

^aDependent Variable: pERK/tERK ratio at diagnosis

^bPredictor: (Constant): WBCs count k/ ul at diagnosis

Table 4. Differences of VCR, ASP, DEX and DOX LCSI % between standard risk (SR) & high risk (HR) groups (gp)

Drug LCSI % (Mean ± SD)	SR gp n = 13	HR gp n = 13	t- test p- value
VCR	24.73±15.41	61.53±22.73	<0.001*
ASP	37.96±16.29	69.46±25.04	0.001*
DEX	40.36 ± 9.54	78.98±20.91	<0.001*
DOX	20.39 ± 5.54	37.61±21.37	0.01*

VCR: vincristine, ASP: l-asparaginase. DEX: dexamethasone, DOX: doxorubicin
 LCSI %: leukemic cell survival index %
 SD = standard deviation
 *p- value is significant if < 0.05

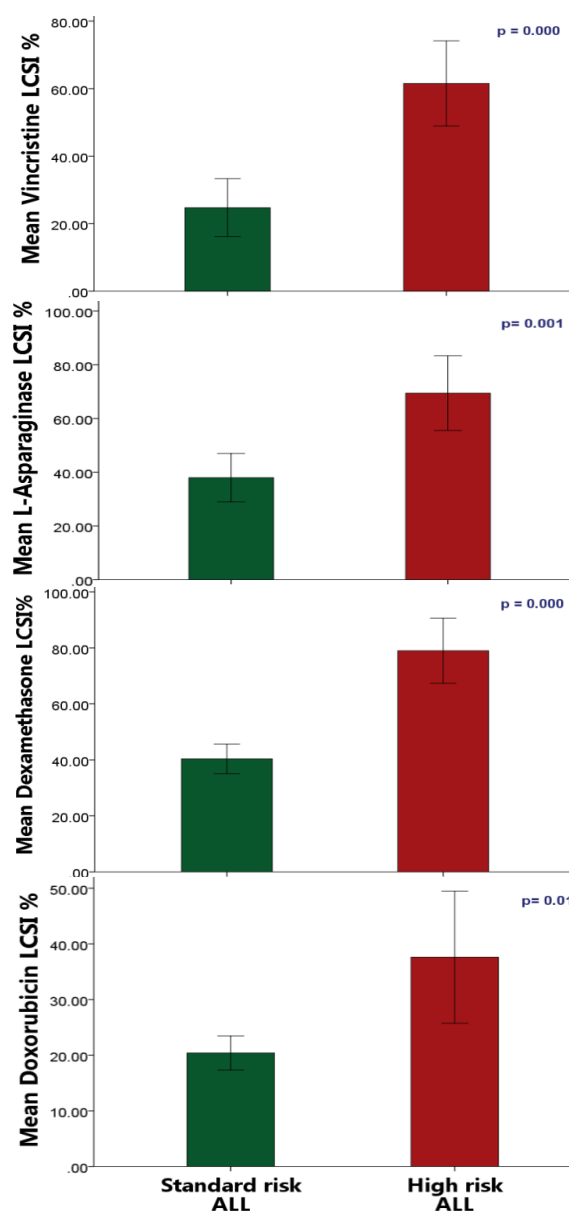


Figure 3. The differences of the four drugs (Vincristine, L-asparaginase Dexamethasone and Doxorubicin) LCSI % between the standard risk (SR) and high risk (HR) ALL groups. (LCSI %: leukemic cell survival index %).

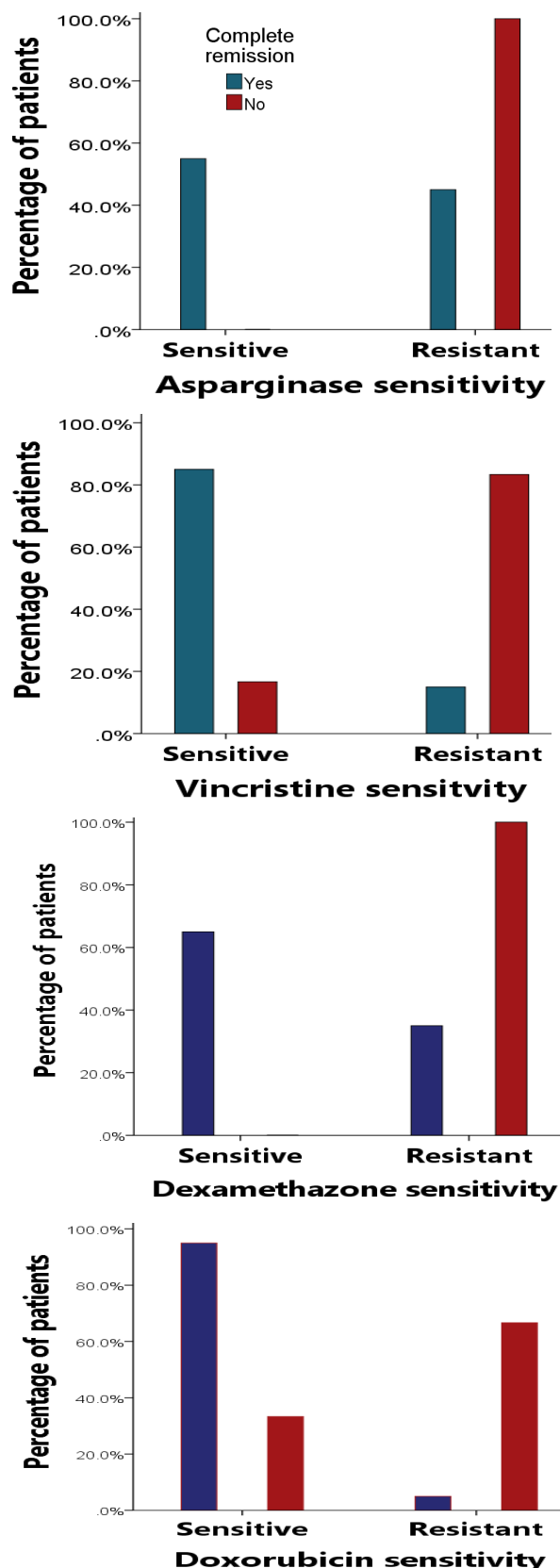


Figure 4. Relation between in vitro chemosensitivity/resistance of (Vincristine, L-asparaginase Dexamethasone and Doxorubicin) & complete remission in 26 children with ALL (Sensitive = LCSI % less than 50% , Resistant = LCSI % more than 50 %).

DISCUSSION

Recognition of oncogenic biomarkers that are constitutively activated in acute leukemia is likely to predict prognosis and permits more characterization of patient subgroups and treatment strategies. The implementation of in vitro chemo-sensitivity tests as a predictive tool in clinical practice is among the most vital future aims in oncology. In our study, we focused on ERK 1/2, a crucial kinase in MAPK pathway for cell proliferation and differentiation. Besides, we performed in-vitro chemo-sensitivity assay for 4 chemotherapeutic agents (vincristine, l-asparaginase, dexamethasone and doxorubicin).

MAPK pathway is supposed to be vital in the development of leukemia. Point mutations, deletions and chromosomal translocations, that cause deterioration in this pathway signaling is among the causes of leukemia pathogenesis (Akin et al., 2021). In Jasek-Gajda et al. (2019) study, ERK1/2 inhibitor (VX-11e) was used to treat various leukemic cell lines for 24 hours. In addition, it was combined with voreloxin (a topoisomerase II inhibitor). The growth of leukemia cells was inhibited by VX-11e; it also affected the cell cycle and induced apoptosis. Furthermore, a synergistic anticancer effect in leukemia cells was employed by VX-11e and voreloxin combination (Mosaad et al., 2017).

In the current study, the pERK and tERK 1/2 detected at diagnosis in bone marrow aspirate samples of patients with high risk ALL were significantly higher than those of standard risk ALL. Our detections could be supported by a number of studies that revealed the strong relation between the mutations in Ras genes & consequently high abnormal proteins and the unfavorable (high risk) criteria in All (Perentes et al., 2004). Our results concerning pERK and tERK 1/2 after induction phase came in divergence to those at diagnosis. There were no significant differences of pERK, tERK 1/2 between the study groups post induction phase. However, the pERK/ tERK 1/2 ratio at diagnosis was significantly higher than that post induction.

A highly significant correlation was detected between pERK/ tERK 1/2 ratio and WBC count at diagnosis. The WBC count was supposed to

be a predictor for pERK/tERK ratio at diagnosis. Interestingly, this came in conformity with Gregorj et al. 2007 study that reported significant association between the expression of pERK1/2 and WBC counts in newly diagnosed ALL. There was a direct correlation between pERK 1/2 higher level and higher WBC count. Moreover, Gregorj and co-workers considered ERK1/2 phosphorylation as an independent predictor of complete remission (Gregorj et al., 2007). In our study, the complete remission was associated with low pERK/tERK 1/2 ratio at diagnosis. Patients with high pERK/tERK 1/2 ratio unfortunately had no complete remission.

Concerning the in vitro chemo-sensitivity in this study, the leukemic cells from children with high risk ALL features showed significantly in vitro resistance to vincristine, l-asparaginase and dexamethasone compared with standard risk group. As regard the in vitro response to doxorubicin, there was a little but significant difference between high and standard risk groups. These results came in harmony with those of (Galderisi et al., 2009) study that showed significantly decreased in vitro sensitivity to vincristine, l-asparaginase and dexamethasone in patients with high-risk features (as high leukemic cell burden or unfavorable genetics). Moreover, the results of the current study came in accordance to those of (Kaspers et al., 1997; Kaspers et al., 1998) studies that revealed a strong relationship between the in vitro cellular resistance to chemotherapeutics and the high-risk factors in newly diagnosed pediatric ALL.

Additionally, in the current study, a significant association was detected between in vitro chemo-sensitivity to vincristine, l-asparaginase, dexamethasone & doxorubicin and complete remission after induction therapy. The strength of this association was intermediate to strong. In vitro drug resistance to each of the four chemotherapeutics agents used was associated with worse outcome after combination therapy and refractory disease. These results came in accordance with those of (Jun et al., 2007) study that predicted the occurrence of complete remission after induction chemotherapy in children with ALL by the aid of the MTT in vitro chemo-sensitivity assay.

Finally, a significant positive correlation was detected between ERK 1/2 (pERK & tERK) and LCS1 % of vincristine, l-asparaginase, dexamethasone and doxorubicin. The correlation was of intermediate strength and pERK 1/2 was considered as predictor for the LCS1 % of the four drugs. A supportive data to our results indicated that the chemotherapy resistant leukemic cells exhibited significantly higher expression of MAPK pathway components. Furthermore, inhibition of MAPK Kinase (1 & 2) enhanced the glucocorticoid-induced cell death. There was an increased in vitro dexamethasone activity in ALL blasts from 19 of 22 tested patients as a result of MEK suppression (Polak ET AL., 2016).

CONCLUSION

To put it briefly, our study showed that in vitro drug resistance of leukemic cells to vincristine, l-asparaginase, dexamethasone & doxorubicin was associated with worse outcome after combination therapy and lack of complete remission. Besides, the ERK 1/2 at diagnosis was significantly higher in the high-risk group than the standard risk group. The complete remission was associated with low pERK/tERK 1/2 ratio. Correspondingly, ERK1/2 could be considered as an independent predictor of complete remission.

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No fund was received.

CONFLICT OF INTEREST

The authors declare no competing financial interests.

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