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TSPAN5: A Potential Biomarker for Methotrexate Resistance in Acute Lymphoblastic Leukemia in Iraq

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Abstract:

The transmembrane receptor family known as tetraspanins (TSPANs) was integral to diverse cellular processes, including protein anchoring, scaffolding, and signal transduction. While the functional repertoire of TSPANs is still being elucidated, their involvement in cancer development and progression is increasingly recognized. Specifically, tetraspanin 5 (TSPAN5) has been implicated in modulating key cellular behaviors such as survival, proliferation, and invasion. Critically, our previous work established a crucial role for TSPAN5 in the development of chemoresistance against conventional chemotherapeutics, including vincristine (VCR), methotrexate (MTX), and doxorubicin (DOXO), in malignant cells and no untreated cases. This study comprehensively reviews and analyzes current understanding of TSPAN5's physiological mechanisms in ALL and CLL, with a particular emphasis on its contribution to chemoresistance. We report a statistically significant upregulation of TSPAN5 in ALL patients stratified by chemotherapy regimen compared with untreated cases, specifically within the cohort treated with MTX ($p < 0.0001$). Although elevated TSPAN5 levels were observed in the VCR-only and DOXO-treated groups, these increases did not reach statistical significance. Furthermore, TSPAN5 expression in untreated CLL patients was comparable to controls; there was no discernible variation in TSPAN5 expression between the ALL and CLL groups. These results offer strong proof that TSPAN5 contributes to chemotherapy resistance in ALL, especially when MTX is used as a treatment. In light of these findings, we talk about the possible ways that TSPAN5 functions in leukemogenesis and suggest that focusing on TSPAN5 might be a useful therapeutic approach for overcoming chemoresistance and enhancing leukemia treatment results.

Key words: TSPAN5, ALL, CLL, DOXO, VCR, and MTX

Introduction

Four transmembrane domains define the Tetraspanins (TSPANs) family of transmembrane proteins, which function as key organizers of cellular membranes (1,2). They orchestrate the formation of molecular complexes that regulate diverse cellular processes, including cell adhesion, migration, and signal transduction (3,4). Among these, Tetraspanin 5 (TSPAN5) has emerged as a particularly intriguing molecule due to its context-dependent roles in various cancers (5,6). While TSPAN5 has been shown to promote Hepatocellular carcinoma (HCC), metastasis, and epithelial-mesenchymal transition (EMT) via Notch signaling (7-9). It paradoxically suppresses tumors in gastric cancer (GC) by modulating cell cycle regulators such as p27 and cyclin D1/CDK4, ultimately inducing senescence (10,11). This functional duality highlights the complex nature of TSPAN5 and its therapeutic potential (12-14).

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Macrophages in the tumor microenvironment (TME) are essential for forming the trajectory of malignancy. Tumor-associated macrophages (TAMs) contribute significantly to angiogenesis, immunosuppression, and metastasis (15,16). However, the specific contribution of TSPANs, and particularly TSPAN5, to the pro-tumorigenic functions of TAMs remains largely unexplored (17,18). While recent studies have demonstrated TSPAN5's broader immunological relevance, including its ability to facilitate CD8+ T cell activation by clustering MHC I molecules (19,20), and the contrasting immunomodulatory effects of other TSPAN family members like CD63 and CD151, the precise role of TSPAN5 in myeloid cells (21,22), especially TAMs, and its implications for hematologic cancers including CLL and ALL, are yet to be elucidated. The therapeutic potential of targeting TSPANs is underscored by the promising preclinical results obtained with TSPAN8-specific CAR-T cells (23,24), further emphasizing the need for a deeper understanding of TSPAN5's function in immune cells (25,26).

ALL and CLL, malignancies of lymphoid origin, are significantly influenced by the TME, where myeloid cells and soluble tumor-derived factors contribute to disease progression and therapy resistance (27,28). Although TSPAN5 has been implicated in cytoskeletal remodeling via MRTF-A/SRF pathways and immune synapse formation (29), its expression and functional relevance in leukemia-associated myeloid cells and peripheral blood mononuclear cells (PBMCs) remain unknown. This knowledge gap is particularly critical given TSPAN5's capacity to regulate both oncogenic signaling pathways and immune effector responses, suggesting its potential as a molecular switch balancing tumor suppression and immune evasion in leukemia (30,31). The purpose of this study is to examine the complex function of TSPAN5 in the complex interplay between leukemia cells, myeloid cell subsets, and tumor-derived factors. We hypothesize that TSPAN5 expression in PBMCs and myeloid cells is modulated by leukemia-derived signals, thereby influencing immune dysfunction and disease progression in ALL and CLL. Utilizing qPCR and ELISA, we will characterize TSPAN5 expression at both the transcriptional and protein levels in primary patient samples. Specifically, this investigation will address the critical question of how tumor-derived factors alter TSPAN5 expression in PBMCs from patients with ALL and CLL.

Methodology

Sample Collection and Processing

Between May 1st, 2024, and September 22nd, 2024, blood samples were taken. Twenty healthy controls, twenty patients with acute lymphoblastic leukemia (ALL), and twenty patients with chronic lymphocytic leukemia (CLL) made up the sixty samples that were examined. When classifying patients, disease activity was taken into account. Diagnosis of ALL and CLL was established by a hematologist at the Cancer Oncology Department of Al-Forat Al-Awsat Hospital (Iraq) following established leukemia classification criteria. For each participant, two samples were collected: one plasma sample and one whole blood sample. Plasma samples were immediately frozen upon gathering. From the entire blood samples, peripheral blood mononuclear cells (PBMCs) were separated for ELISA and RNA extraction (qRT-PCR). PBMCs' TSPAN5 mRNA levels were measured by qRT-PCR, and plasma samples' TSPAN5 protein levels were measured using ELISA.

Peripheral Blood Mononuclear Cell Isolation (PBMCs)

Using a Ficoll density gradient, peripheral blood mononuclear cells (PBMCs) were separated from whole blood. centrifugation, following the manufacturer's protocol (Solar-Bio, Lot No. P4350) with minor modifications. In a 50 mL conical tube, 2 mL of whole blood was diluted with 2 mL of sterile phosphate-buffered saline (PBS). Using a Pasteur pipette to reduce mixing, 2 mL of Ficoll-Hypaque

was carefully deposited below 2 mL of the diluted blood in a separate 50 mL conical tube. To get the best separation of blood components, the tubes were subsequently centrifuged at 1400 rpm for 40 minutes at room temperature without the brake. Four separate layers were produced by this process: red blood cells (bottom), plasma (top), PBMCs (middle), and Ficoll-Hypaque. Using a sterile Pasteur pipette, the PBMC layer—a thin, white band at the interface between the plasma and Ficoll-Hypaque—was meticulously extracted and moved to a fresh 50 mL conical tube. In order to eliminate any remaining Ficoll-Hypaque or contaminating cells, the obtained PBMCs underwent two PBS washes. To pellet the cells, the tubes were centrifuged at 300g for 10 minutes after each wash. After carefully removing the supernatant, the PBMC pellet that resulted was resuspended in 1 milliliter of freezing medium, which is FSC media with 5% dimethyl sulfoxide (DMSO) added. An automatic cell counter (Bio-Rad) was then used to count the cells in the cell suspension. The freezing medium was used to modify the PBMC suspension to a final concentration of 2 x 10^6 cells/mL based on the cell count. The produced PBMC solution was then cryopreserved in liquid nitrogen until it could be used in quantitative polymerase chain reaction (qPCR) and other downstream processes.

RT-PCR: -

To create separate experimental cohorts, peripheral blood mononuclear cells (PBMCs) were isolated from patients in remission, patients with active illness, and healthy controls. Using the Solarbio Life Science RNA extraction kit, total RNA was isolated from each sample in accordance with the manufacturer's instructions. In short, cells were lysed, centrifuged (200 x g, 5 minutes), and their RNA was isolated using two washes with WT solution and one wash with RPE buffer. After being rinsed, the purified RNA was kept at -20°C until it was processed further. The process of reverse transcription was used to create complementary DNA (cDNA). The Primer Design Precision 2x qPCR SYBR Green Master Mix was used to conduct quantitative PCR (qPCR) using an Applied Biosystems 7900HT Fast Real-Time PCR System. Targeting the TSPAN5 gene, pre-made, verified primer/probe sets (Macrogen, South Korea) (Forward: 5'-CAGAGGATGTCCGGGAAGC-3'; Reverse: 5'-GATGTTGGACAGAACTCCTTTTTCA-3') and the GAPDH reference gene (Forward: 5'-TGCACCACCAACTGCTTAGC-3'; Reverse: 5'-GGCATGGACTGTGGTCATGAG-3') were employed. 40 cycles of amplification were performed. The comparative cycle threshold (Ct) approach was used to calculate the relative expression of the TSPAN5 gene (32). returning to the expression of GAPDH.

The Estimation of Human TSPAN5

Plasma samples underwent analysis for TSPAN5 concentrations via an immediate assay following a brief centrifugation step to remove particulate matter. A 4000 ng/mL stock standard solution was prepared by reconstituting the standard with 1.0 mL of standard sample diluent. Subsequent serial dilutions were performed by transferring 500 µL of standard sample diluent into a designated 2000 ng/mL tube and serially diluting into the remaining tubes to generate a standard curve. The assay commenced with filling each well of a microtiter plate with 100 µL of the standard or sample. After being sealed and incubated for 120 minutes at 37°C, each well underwent three aspiration and replenishment washes with 1x wash buffer. Each well was then filled with 100 µL of a working solution that contained biotin-conjugated anti-human TSPAN5 antibodies. The wells were then incubated for 45 minutes at 37°C. Three further washes with 1x wash buffer were carried out. Once the adhesive seal on the microtiter plate has been replaced, 100 µL of HRP-avidin working solution was introduced into each well. After three further washes with 1x wash buffer, the plate was incubated at 37°C for 30 minutes. 100 µL of TMB substrate was added to

each well to start the chromogenic reaction. Each well was then gently mixed and incubated for 15 to 20 minutes at 37°C in a dark atmosphere. The reaction was terminated by the addition of 50 µL of stop solution to each well. Optical density measurements were acquired within 30 minutes using a microplate reader set to 450 nm. The TSPAN5 concentrations in the unidentified samples were ascertained by interpolating against the produced standard curve after data collection and analysis.

Statistical Analyses

GraphPad Prism 10 was utilized for all statistical analyses and graphical data visualization. Intergroup comparisons were performed using one-way ANOVA, as appropriate for the experimental design. Post-hoc testing is then performed to account for numerous comparisons. The mean ± SEM is used to express the data.

Results

Gene Expression of TSPAN5 in ALL and CLL treated and untreated patients

To identify whether TSPAN-5 in ALL and CLL cases was up- and/or down-regulated after treatment with different types of chemotherapy compared with untreated cases, we performed a qPCR assay in all patients who suffered ALL and CLL, treated and untreated with chemotherapy, using PBMC cells for expression studies. We selected the patients under treatment course and untreated, either ALL or CLL, compared with the gene level with control healthy cases. The expression of TSPAN5 was upregulated in PBMCs cells for the untreated patient who suffered ALL, in contrast to the treated patients and control group (Figure 1A). However, Figure 2A shows no change in the TSPAN5 expression in CLL patients. TSPAN5 was also shown to be upregulated at the mRNA level of patients during treatment with MTX rather than other chemotherapies, which are VCR and DOXO (Figure 1A). Interestingly, TSPAN-5 was significantly increased in the ALL cases according to the type of chemotherapies, with MTX (P-value 0.0001 ****), the group treated with VCR only was non-significant, and the group treated with DOXO (P-value 0.052), compared with the untreated ALL cases. However, it was non-significant in the CLL group compared with control cases. These changes could be because TSPAN-5 is playing a role in ALL cases rather than CLL. Interestingly, different chemotherapies affect the level of TSPAN-5 at the mRNA level, which could explain the regulatory role of TSPAN5 in cell signaling, including proliferation, apoptosis, and cell generation. VCR&MTX has a high effect on the TSPAN-5 level in the B and T cells of patients.

Figure 1A

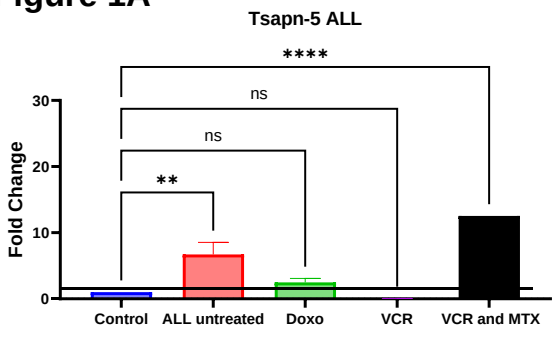


Figure 2A

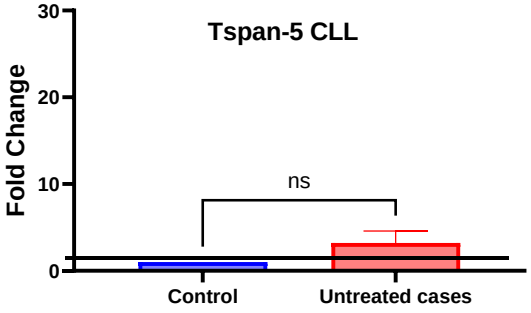


Figure 1A: The level of TSPAN 5 at mRNA in patients who were diagnosed with ALL during treatments compared with the level of this cell surface protein in healthy cases.

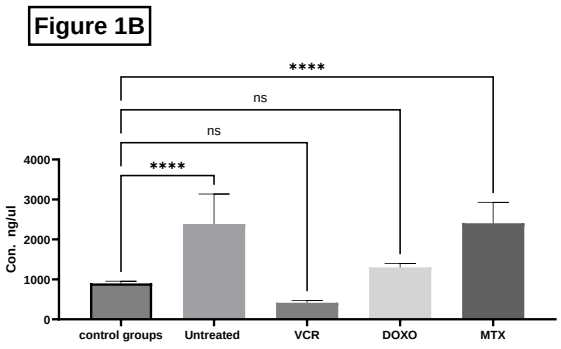
Figure 2A: The level of TSPAN 5 at mRNA in patients who were diagnosed with CLL compared with the level of this cell surface protein in healthy cases.

The TSPAN5 expressions were measured by qPCR in patients with ALL and CLL. After estimating the housekeeping gene GAPDH, the 2-ΔΔCt technique was used to quantify TSPAN5 expression. TSPAN5 revealed alterations in ALL in the patients' PBMCS cells. One-way ANOVA was used to evaluate the significance of differences, with ns=non-significant and **** p<0.0001 significant. The statistics represent the averages of three different studies for eighty patients, each with a duplicate.

Tspan-5 Expression is Upregulated in ALL but not CLL

This study investigated the hypothesis that Tspan-5 levels are altered in ALL and CLL Plasma samples from healthy controls and patients with ALL or CLL, stratified according to pre-defined clinical criteria, were gathered and kept in storage at -20°C. A commercial sandwich ELISA kit (Solorbio, China) was used to measure the TSPAN5 concentrations in the plasma sample in accordance with the manufacturer's instructions. Standard curve analysis was used to accomplish quantification. The plasma TSPAN5 levels in ALL patients were shown to be statistically significantly higher than those in healthy controls (Figure 2B). TSPAN5 levels in CLL patients and healthy controls did not differ significantly. Crucially, treatment naïve ALL patients showed a more marked rise in TSPAN5 ($p < 0.0001$) than the control group ($p < 0.01$). Additionally, compared to patients receiving vincristine (VCR) or doxorubicin (DOXO), ALL patients receiving methotrexate (MTX) also had higher levels of TSPAN5 mRNA (Figure 1B). When TSPAN5 protein levels in ALL patients were analyzed by chemotherapy regimen, the group receiving MTX showed a substantial increase ($p < 0.0001$). When compared to untreated ALL patients, TSPAN5 levels were substantially higher in the DOXO-treated group ($p = 0.072$) but not statistically different in the VCR-treated group.

TSPAN5 protein ELISA has examined expression levels in patients with ALL and CLL in comparison to healthy individuals. After estimating manufacturer information, the standard curve approach was used to calculate the expression of TSPAN5 in both healthy and non-healthy instances. TSPAN5 showed changes in plasma. One-way ANOVA was used to evaluate the significance of differences; **** p<0.0001 is significant, * p<0.05 is significant, and ns is non-significant. The means of 80 samples from three different tests with duplicates make up the data.



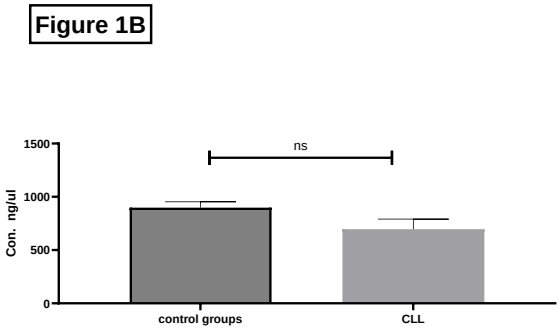


Figure 1B: The level of TSPAN 5 at the protein level in patients who were diagnosed with ALL during treatments compared with the level of this cell surface protein in healthy cases.

Figure 2B: The level of TSPAN 5 at the protein level in patients who were diagnosed with CLL compared with the level of this cell surface protein in healthy cases.

Discussion

TSPAN5 Upregulation in Acute Lymphoblastic Leukemia and Its Implication in Chemotherapy Response

The function of TSPAN5, a tetraspanin superfamily member, in ALL and CLL was examined in this work. We hypothesized that TSPAN5 levels are altered in these hematological malignancies. Plasma samples from well-characterized ALL and CLL patients, alongside healthy controls, were analyzed, with patient groups stratified according to established clinical criteria. Our findings reveal a statistically significant upregulation of TSPAN5 protein levels in ALL patients treated with a combined regimen of vincristine (VCR) and methotrexate (MTX) compared to untreated ALL cases ($p < 0.0001$). While elevated TSPAN5 levels were observed in ALL patients treated with VCR alone or doxorubicin (DOXO), these increases did not reach statistical significance (Figure 1A and 2A). Importantly, TSPAN5 expression in untreated CLL patients was comparable to controls, and no significant difference in TSPAN5 expression was observed between the ALL and CLL groups. Furthermore, our data suggests a potential link between TSPAN5 and chemotherapy resistance in ALL, particularly in the context of MTX treatment (Figure 1B and 2B).

These observations align with previous reports demonstrating TSPAN5 and N-cadherin (CDH2) upregulation upon DLC1 depletion in hepatocellular carcinoma (HCC) cells (32-34). We postulate that the increased TSPAN5 levels in ALL may reflect a dysregulated mononuclear phagocyte system, a key feature of disrupted immune tolerance. This heightened TSPAN5 production could, in turn, influence T and B cells, as well as other immune cells, potentially contributing to hemolysis, a common complication of ALL. These recruited immune cells may further exacerbate red blood cell destruction through the release of inflammatory mediators (35,36).

Previous work has also suggested a role for TSPAN5 in cell migration. Knockdown of TSPAN5 in macrophage-derived macrophages (MDMs) has been associated with increased cell migration in vitro (37,38). While the specific role of TSPAN5 in cell migration remains to be fully elucidated, other tetraspanin family members have been implicated in this process. For instance, CD82 has been shown to inhibit prostate cancer cell migration by modulating actin dynamics and stress fiber formation (39), whereas CD151 promotes cell migration through integrin trafficking (40).

Although limited data exists regarding TSPAN5 in ALL and CLL, other tetraspanins have known roles in hematopoiesis and immune function. CD81 interacts with CD19 in B-cell receptor signaling and also plays a role in T cells (41,42). CD231 (TALLA-1/Tspan7) is expressed in T-lymphoid leukemic and neuroblastoma cell lines (43). CD9, often co-expressed with CD81 or CD53 in B-cell precursor ALL and clonal plasma cells, has been used as a marker in leukemia diagnosis (41).

Based on these findings, we propose a model where TSPAN5 contributes to ALL pathogenesis, particularly in untreated patients and those exhibiting chemoresistance. Our data suggest that TSPAN5 plays a significant role in the immune response in ALL, and potentially in CLL. Elevated TSPAN5 protein levels, particularly in the MTX-treated group, correlate with disease characteristics and may serve as a potential biomarker for ALL. Further investigation is warranted to dissect the precise mechanisms by which TSPAN5 contributes to ALL pathogenesis, including its role in hemolysis and chemoresistance. Targeting TSPAN5 may represent a novel therapeutic strategy for improving outcomes in leukemia treatment.

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Declaration

The authors have no conflicts of interest to declare, and the financial support is University of Kufa.

Ethics Clearance

This research was approved to use human samples from patients and approved to contain these samples by the MOH committee for ethics in Iraq with legal paper 1035, 24 May 2024.

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