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The Impact of *Hsa-circ-0001947* on TGF-β Signaling in Iraqi Patients with Acute Myeloid Leukemia

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Abstract

Acute myeloid leukemia (AML) is a hematologic malignancy characterized by the accumulation of immature myeloid cells in the bone marrow, leading to impaired hematopoiesis. Circular RNAs (circRNAs) have emerged as key regulators in gene expression and cancer progression, including AML. This study investigates the expression of circRNA (*hsa_circ_0001947*) and its correlation with Transforming Growth Factor - β (TGF-β) serum levels in Iraqi AML patients. A cohort of 50 patients with AML and 40 healthy individuals was examined. The *hsa_circ_0001947* expression was analyzed using quantitative RT-PCR, while serum TGF-β levels were measured using the ELISA technique. The results demonstrated a significant downregulation of *hsa_circ_0001947* in AML patients compared to healthy controls ($P \leq 0.05$). Additionally, AML patients exhibited significantly lower TGF-β levels ($P = 0.0001$), with a positive correlation ($r = 0.3035$, $P \leq 0.05$) between *hsa_circ_0001947* expression and TGF-β levels. Subgroup analysis indicated that hsa_circ_0001947 expression was lowest in newly diagnosed and relapsed AML patients compared to those undergoing treatment. These findings suggest that *hsa_circ_0001947* may play a regulatory role in AML progression and could be linked to immunomodulatory mechanisms involving the TGF-β pathway. Further research is warranted to explore the molecular mechanisms underpinning *hsa_circ_0001947* function in AML and its potential as a prognostic biomarker or therapeutic target.

Keywords: Acute Myeloid Leukemia, CircRNA(*hsa_circ_0001947*), TGF-β, Gene Expression, Immunomodulation, Leukemogenesis.

تأثير التعبيرالجيني لـ *hsa_circ_0001947* على اشارات الـ TGF−β لدى المرضى العراقيين المصابين بسرطان الدم النقوي الحاد

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الخلاصة

ابيضاض الدم النقوي الحاد (AML) هو سرطان دموي خبيث يتميز بتراكم الخلايا النقوية غير الناضجة في نخاع العظم، مما يؤدي إلى ضعف تكوين خلايا الدم. برزت الحمض النووي الريبوزي الدائري (circRNAs) كمنظمات رئيسية للتعبير الجيني وتطور السرطان، بما في ذلك ابيضاض الدم النقوي الحاد. تبحث هذه الدراسة في تعبير الحمض النووي الريبوزي الدائري (*hsa_circ_0001947*) وارتباطه بمستويات TGF−β في مصل الدم لدى المرضى العراقيين المصابين بأبيضاض الدم النقوي الحاد. تم فحص مجموعة من 50 مريضًا مصابًا بابيضاض الدم النقوي الحاد و40 فردًا سليمًا. تم تحليل تعبير *hsa_circ_0001947* باستخدام تفاعل البوليميراز المتسلسل العكسي الكمي(RT−PCR) ، بينما تم قياس مستويات TGF−β في مصل الدم باستخدام تقنية الـ ELISA. أظهرت النتائج انخفاضًا ملحوظًا ($P \leq 0.05$) في التعبير الجيني لـ *hsa_circ_0001947* لدى مرضى ابيضاض الدم النقوي الحاد مقارنةً بالأصحاء. بالإضافة إلى ذلك، أظهر المرضى مستويات−TGF βمنخفضة بشكل ملحوظ ($P = 0.0001$) ، مع وجود ارتباط إيجابي ($r = 0.3035$ ، $P \leq 0.05$) بين التعبير الجيني لـ *hsa_circ_0001947* ومستويات TGF−β. أشار تحليل المجموعة الفرعية إلى أن التعبير الجيني لـ *hsa_circ_0001947* كان في أدنى مستوياته لدى مرضى ابيضاض الدم النقوي الحاد المُشخَّصين حديثًا والمُصابين بانتكاسة مقارنةً بمن يخضعون للعلاج. تشير هذه النتائج إلى أن *hsa_circ_0001947* قد يلعب دورًا تنظيميًا في تطور ابيضاض الدم النقوي الحاد، ويمكن ربطه بآليات تعديل المناعة التي تتضمن مسار TGF−β.هناك حاجة إلى مزيد من البحث لاستكشاف الآليات الجزيئية التي تدعم وظيفة *hsa_circ_0001947* في ابيضاض الدم النقوي الحاد، وإمكانية استخدامه كمؤشر حيوي تشخيصي أو هدف علاجي.

1. Introduction

Acute myeloid leukemia (AML) displays characteristics of aggravated blood and bone marrow cancer when clonal myeloid progenitor cells remain undeveloped into mature blood cells, which is accompanied by decreased blood counts across multiple lines [1,2]. AML makes up almost 80% of all cases of leukemia and is the most prevalent kind in adults. In children, it is responsible for 15-20% of leukemia cases, however, in adolescents and young adults, it accounts for roughly 33%. The accumulation of mutations from genetic error events in cell division and extended exposure to environmental carcinogens are linked to the increased incidence of AML in older age groups, particularly those over sixteen [3,4].

Circular RNAs (circRNAs) are indeed non-coding RNA (ncRNAs) and they are generated by ligating the 5 ‘and 3 ‘ends of a single-stranded RNA molecule and connecting end-to-end to form a closed circle. CircRNAs have been identified to be involved in the regulation of gene expression, inhibition of protein function, and protein coding. Recent computational analyses combined with next-generation sequencing (NGS) have pointed to the fact that many circRNAs are rich and preserved evolutionarily among species [5,6]. CircRNAs that possess several microRNA (miRNA) binding sites have the ability to function as miRNA sponges. Some abundant circRNAs may connect with miRNAs by complementarity, allowing mRNA translation by removing miRNAs from target mRNAs [7]. There are multiple roles of circular RNAs in acute myeloid leukemia. Damage may cause deregulation of circRNA, which plays a role in the progression of AML and leukemic stem cell resistance to chemotherapy [8].

Transforming Growth Factor - β (TGF-β) is identified to have the ability to act as an inhibitory growth factor in the preliminary stages of carcinogenicity and prevents the formation of tumors. However, surprisingly, it reverts back to a tumor promoter at later stages, and contributes to metastatic progression by autocrine TGF-β loop [9]. In blood cancers (hematologic malignancies), one common change is that the cancer cells stop responding to TGF-β, a protein that normally controls cell growth, development, and death. Unlike solid

tumors, blood cancers usually become resistant to TGF-β by making fewer TGF-β receptors or by blocking its signals using certain cancer-related proteins like Evi-1 and Tax. On the other hand, solid tumors often have mutations in the TGF-β signaling pathway itself, such as in the *Smad4* or *TβRII* genes [10]. Leukemic cells also show differential expression of two isoforms of TGF-β Receptor II (TβRII), which are TβRII and TβRII-B as compared to normal hematopoietic cells. Only TβRII-B was observed for inducing apoptosis, arrest of the cell cycle, and leukemic cells differentiation, not TβRI. TβRII-B also enhanced the binding of TGF-β1 and the transduction of subsequent signals and suppressed the tumor formation in vivo. However, TβRII was against the induction caused by all-trans retinoic acid to promote differentiation by interfering with TβRII-B [11].

This study aims to investigate the fold expression of *hsa_circ_0001947* in peripheral blood of AML patients and healthy subjects and to correlate their expression with TGF-β serum levels and explore the potential role of circRNA (*hsa_circ_0001947*) as a biomarker for AML diagnosis, prognosis, and treatment response.

2. Materials and Methods

Subject of the study and blood sampling

This study included 50 patients with AML (28 males and 22 females) attending the Hematology Center in Medical City of Baghdad during the period from December 2023 to April 2024. Forty family-unrelated healthy individuals (22 male and 18 female) were selected to represent the control group. The AML patients were divided into three groups: newly diagnosed (ND), undergoing treatment (UT), and relapse (R) group. About five ml of venous blood was collected from each participant; 2 ml was kept in an EDTA tube (300 µl of it was transferred to 600 µl of Trizol reagent in an Eppendorf tube). The other 3 ml was put in the gel tube and left to clot. Then serum was obtained in a plain tube (for ELISA tests). This study was approved by the University of Baghdad's College of Science Ethics Committee (Ref.: CSEC/0724/0048).

Extraction of RNA and Synthesis of cDNA

Total RNA (including circRNA) was isolated from blood samples using the ready kit (TransZol Up Plus RNA kit/Transgene/China) by adopting the manufacturer's protocol. Quantus™ Fluorometer was used for evaluating the purity of extracted RNA. The total RNA was converted into cDNA by using the Easy Script® one-step gDNA removal and cDNA synthesis superMix Kit procedure. RT-PCR was achieved by using a specific primer made by Macrogen (Korea) in a lyophilized form.

Primer design

The primer sequences for CircRNA and the housekeeping gene (*U6*) used in this study are shown in Table 1.

Table 1: Sequences of the primers in this study

Name of primers	Sequences (5'⟶3')	TM
hsa_circ_0001947	F: ACAAGTGAAACAAACAAAGGTGA	57.54
	R: TCCAAGCGTGTTCTGGACTC	59.97
U6	F: GACCTGCTCTGGTGGTCTTG	60.32
	R: GAACCACACTCTGGGACAGG	59.96

Performing Real-Time PCR and gene expression calculation

Quantitative RT-PCR (qPCR) using the *PerfectStart*® Green qPCR SuperMix. The reaction mixtures have a total volume of 20 µl, comprising 0.5 µl of primers, 10 µl of PerfectStart Green

qPCR SuperMix (2X), 0.4 µl of passive reference dye, 4 µl of cDNA from each sample, and 4.6 µl of nuclease free water. Real-time-PCR was applied for duration of 30 seconds at a temperature of 94 °C in order to initiate the activity of the polymerase enzyme. Subsequently, the double-stranded cDNA was denatured for 5 seconds at 94 °C and then annealed for 35 seconds at 60 °C using channel scanning, repeating this process 45 times. Finally, the dissociation step for 15 seconds at 60-90 °C. The Ct value of *hsa_circ_0001947* was normalized using the U6 housekeeping gene. The expression of *hsa_circ_0001947* was assessed using a relative quantitative technique, which involved the comparative Ct formula and the 2^-ΔΔCT analysis [12,13].

TGF-β serum levels detection

Serum TGF-β levels were detected for AML patients and healthy controls using the SUNLONG Company *in vitro* Sandwich-ELISA kit.

Statistical analysis

The study parameters underwent analysis using statistical analysis system (2023). It measured the impact of patient and control groups through the T-test and least significant difference (LSD). The percentages (0.05 and 0.01 probability) were compared significantly using the chi-square test, the study's estimate of the correlation coefficient between the variables [14,15].

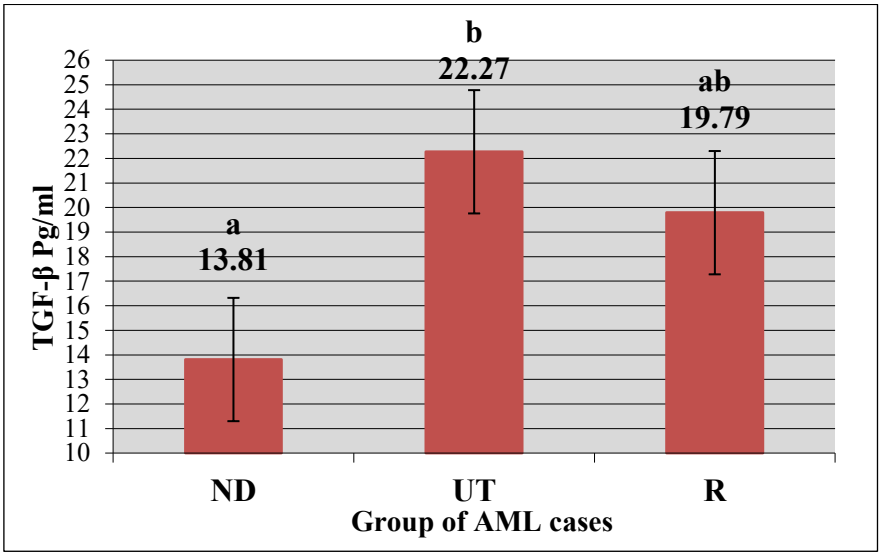
3. Results and discussion

The result of the mean of TGF-β level in serum patients with AML (17.86 ±1.67 pg/ml) was significantly (P=0.0001) reduced when compared with healthy controls (47.93 ±3.63 pg/ml). This result agreed with Wang *et al.,* and Luciano *et al.,* who reported that the serum TGF-β level was significantly decreased in AML patients when compared with healthy volunteers [16,17]. Both CD4+ regulatory T-lymphocytes (Tregs) and regulatory B-lymphocytes (Bregs) exhibit particular immune control features in limiting excessive immunological responses. It could repress inflammation and autoimmunity and support tumor progression. TGF-β and IL-10 are key modulators in immune tolerance and in the functions regulating Tregs and Bregs. TGF-β levels were lower, and plasma IL-10 concentrations were increased in AML patients, indicating a disruption in immunological homeostasis [18]. TGF-β is crucial for controlling hematopoietic progenitor cell differentiation and growth suppression. TGF-β has two functions: it promotes tumor growth in carcinoma cells and suppresses tumor growth in premalignant cells. It functions as a tumor suppressor in the early phases of carcinogenesis by downregulating MYC expression and using cyclin-dependent kinase (CDK) inhibitors to prevent proliferation and encourage apoptosis. The TGF-β suppressive role on leukemic blasts has been described in a large age-independent population of AML with significantly lower TGF-β levels [19].

A distorted TGF-β signaling pathway, when combined with weakened TGF-β protein levels facilitates cancer development from leukemia. Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) and SCF along with Interleukin 1,3 and 6 (IL-1, IL-3 and IL-6) contain surface receptors that TGF-β either blocks or interferes with, thereby inhibiting the signaling pathways activated by these cytokines. Decreased signaling control of TGF-β, together with its suppressed levels, creates an unequal cytokine network that affects the movement, reproduction, and sustenance of cells [19]. TGF-β levels were found to be lower by Wu *et al.* in AML patients, demonstrating that Smad5β manifestation abnormalities within erythroleukemia TF1 cell lines modify TGF-β/BMP ligand-triggered erythroid differentiation responses. Two types of mutations in the TGF-β signaling pathway disable *Smad4* through a

P102L missense mutation in the mad homology 1 (MH1) domain along with a Δ (483-552) frameshift mutation in the mad homology 2 (MH2) domains. This leads to AML [20].

The comparison between the TGF-β levels among different types of AML demonstrated a significant difference (P<0.05) between ND (13.81 ±2.72 pg/ml) and UT (22.27 ±2.08 pg/ml), with insignificant statistical difference between both groups and R patients (19.79 ±3.29 pg/ml) (Figure 1).



ND: Newly diagnosis, UT: Undergoing-treatment, R: Relapse. Different letters mean significant differences between columns

Figure 1: Serum TGF-β levels among different groups of AML patients

Wu *et al.* also reported that serum TGF-β1 concentrations in patients with leukemia infection were significantly decreased compared with healthy controls, but reverted to normal in those who achieved total remission and dropped when patients relapsed [21]. T-helper 17 (Th17) differentiation process may be significantly influenced by TGF-β1. ND patients' bone marrow microenvironment had significantly fewer Th17 cells than that of complete remission (CR), relapse-refractory patients, or controls. The significance of Th17 in AML pathogenesis is demonstrated by the partial correction of decreased Th17 frequencies following standard chemotherapy. Th17 cell reduction in ND AML patients could be attributed to a weakened immune response, but the Th17 cell rise in CR patients suggested an immune system protective response to chemotherapy [22].

Amplification program of real-time PCR was carried out using melting curve analysis for *hsa_circ_0001947* gene in accordance with the manufacturer's simulation program using relative gene expression (Figure 2).

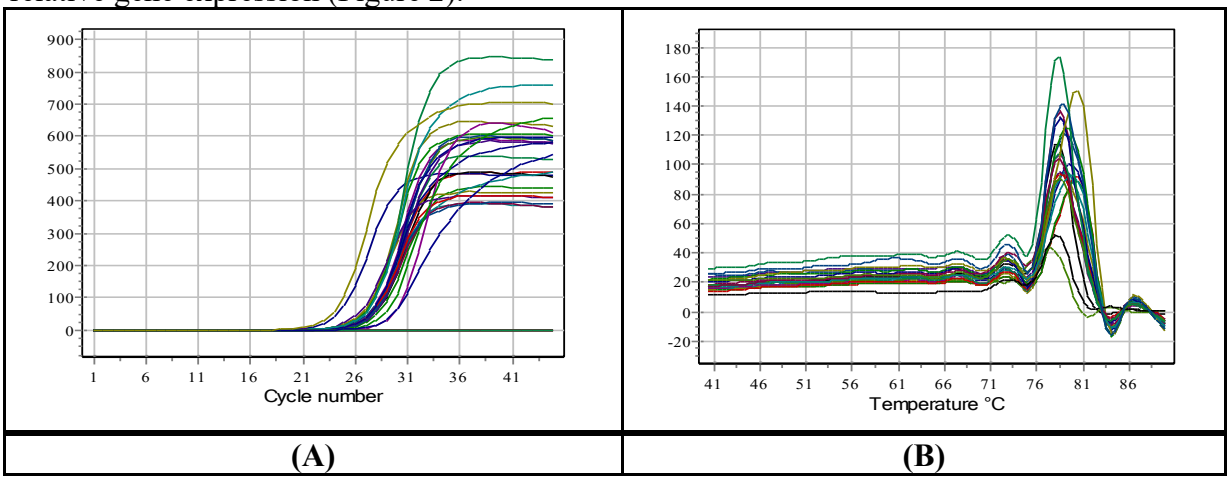


Figure 2: Expression of cricRNA (*hsa_circ_0001947*) (A) CT value (B) Melting curve

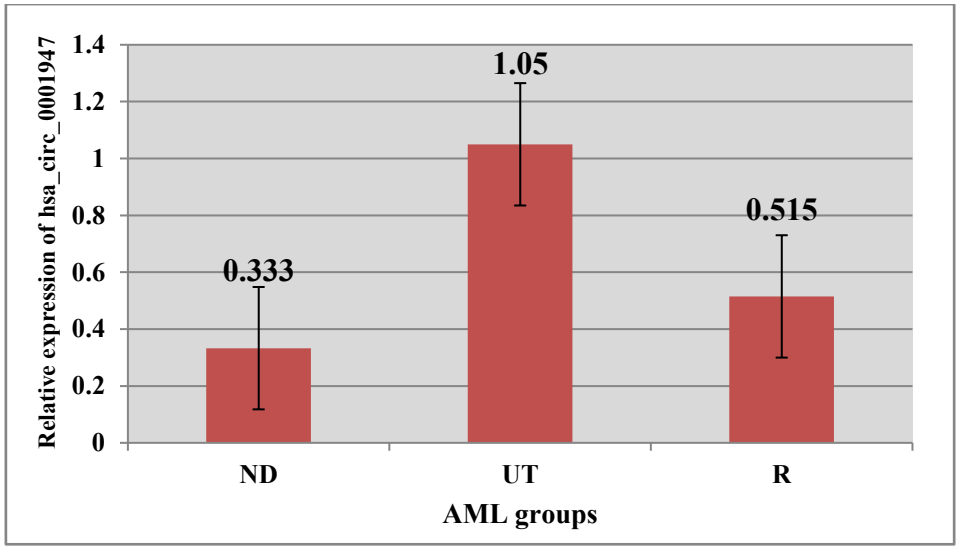
According to the analysis of *hsa_circ_0001947* expression data after normalization with *U6* snRNA gene, the *hsa_circ_0001947* levels were significantly (P≤0.05) down-regulated in AML patients (0.584 ±0.080) compared with healthy controls (1.00 ±0.00), as shown in Table 2.

Table 2: Gene expression and fold change of *hsa_circ_0001947* in patients and controls

Group	hsa_circ_0001947***	ΔCT***	ΔΔCT***	2^-ΔΔCT***	Fold change***
AML Patients	28.004 ±0.332	8.616 ±0.337	1.016 ±0.337	1.041 ±0.142	0.584 ±0.080
Control	26.537 ±0.287	7.610 ±0.363	0.010 ±0.363	1.785 ±0.337	1.00 ±0.00
P-value	0.0033**	0.0559NS	0.0559NS	0.0223*	0.0223*

** (P≤0.05), ** (P≤0.01), *** Mean ±SE, NS: Non-Significant.*

The *hsa_circ_0001947* expression level in newly diagnosed (0.326 ± 0.068) and relapsed AML patients (0.515 ± 0.178) was significantly (P≤0.01) much lower compared to that in undergoing-treated patients (1.050 ± 0.134) (Figure 3).



ND: Newly diagnosed, UT: Undergoing treatment, R: Relapse.

Figure 3: *hsa_circ_0001947* expression among AML patient groups

This result was in agreement with Han *et al.*, who reported that *hsa_circ_0001947* was down-regulated in AML patients when compared with healthy controls, and the expression in ND and R was much lower compared to that of complete remission. They also showed that *hsa_circ_0001947* has the potential to be a promising prognostic biomarker in AML, and it suppressed tumor growth by preventing cell division [23].

CircRNAs modify biological cell operations through their interaction with signaling pathways. The abnormal expression of numerous circRNAs has been identified in many different types of cancer tissue. The RNA molecules function as ceRNA (competitive endogenous RNA) to soak up particular miRNAs, which creates circRNA-miRNA-mRNA interaction networks. CeRNA network irregularities can induce cellular pathway dysfunctions that control apoptosis-associated genes before leading to cancer advancement [24].

Notably, several investigations have demonstrated that AML patients exhibit reduced *hsa_circ_0001947* expression due to *miR-329-5p* binding, which simultaneously limits cell growth while promoting cell death [25-27]. Results showed that the restoration of *CREBRF*

occurred through the expression of *hsa-miR-329-5p* target gene by *hsa_circ_0001947*. A circRNA-miRNA-mRNA interaction network operating in AML research confirmed *hsa_circ_0001947* as an important factor in leukemia development [23]. The *hsa-miR-329-5p* gene is linked to pathways related to apoptosis (like the cell cycle and nucleotide excision repair pathway), metabolism (like the proline and arginine metabolism pathway and the biosynthesis of steroids), and immunity (like the toll-like receptor signaling pathway) [28]. According to an additional investigation, CREBRF effectively suppressed tumors in glioblastoma by preventing hypoxia-induced autophagy through the pathway of CREB3/ATG5 [29].

Hu *et al.* reported that *hsa_circ_0001947* was significantly downregulated while *hsa-miR-454-3p* was upregulated in AML individuals with inverse correlation, they speculated that circRNA (*hsa_circ_0001947*) through miRNA (*hsa-miR-454-3p*) promoted the development of leukemia and forming an association genes network between circular RNA, micro RNA, and hub genes, including 3 regulatory axes: *hsa_circ_0001947/hsa-miR-454-3p/CREB5* axis, *hsa_circ_0001947/hsa-miR-454-3p/BMP6* axis, *and hsa_circ_0001947/hsa-miR-454-3p/TGFBR2* axis [30]. However, functional studies demonstrated that miR-454-3p targets ZEB2 and modifies the AKT/mTOR signaling pathway to induce death and autophagy in AML cells. This emphasizes how it suppresses tumors in AML [31,32].

Figure (4 showed a significant correlation (r=0.3035/ P≤0.05) between *hsa_circ_0001947* expression and TGF-β serum level.

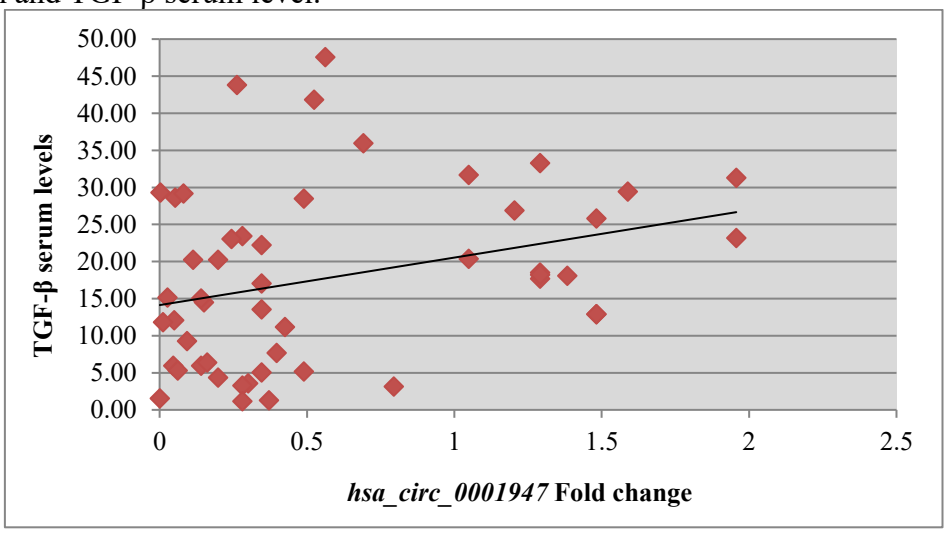


Figure 4: Pearson correlation coefficient between *hsa_circ_0001947* fold change and TGF-β serum levels in AML patients

There are no previous studies that demonstrated the direct interaction between *hsa_circ_0001947* and the TGF-β signaling pathway in AML. According to Ranjbary and KajiYazdi, TGF-β1 serum levels and mRNA expression of TGF-β1 in peripheral blood mononuclear cells were both significantly decreased in AML patients [33]. However, leukemia cell multidrug resistance forms through hypoxia activation of TGF-β/Smad2 signaling and miR-425 acts as a prognostic factor in AML while SMAD2 serves as a predicted target of miR-425. The binding of DARS-AS1 to *miR-425* increases TGFB1 expression, which enables AML disease progression through Smad2/3 signaling. Extensive changes exist in ILCregs (regulatory innate lymphoid cells)-related gene miRNA datasets that include TGFBR2 in AML patients [30].

MicroRNA (*miR-454-3p*) binds tumor suppressor gene WTX, through which it controls TGF-beta signaling mechanisms in hepatocellular carcinoma (HCC). WTX loss results in TGF-β signaling stimulation along HCC progression through miR-454-3p-mediated effect. The miR-454-3p/WTX/TGF-β signaling pathway provides fresh prospects for detecting and treating HCC [34].

Furthermore, by targeting TGF-β2, a ligand of the TGF-β pathway, *miR-454-3p* has been demonstrated to inhibit cell proliferation and metastasis in non-small cell lung cancer (NSCLC). Because *miR-454-3p* was overexpressed in NSCLC cells, TGF-β2 expression was downregulated, which inhibited the epithelial-mesenchymal transition (EMT) and hindered the cells' ability to spread [35].

Conclusion

This study highlights the downregulation of *hsa_circ_0001947* in patients with AML and its positive correlation with TGF-β serum levels, suggesting a potential role of this circRNA in AML pathogenesis. The significantly lower TGF-β levels in patients with AML compared to controls reinforce its involvement in leukemogenesis and immune regulation. Moreover, the differential expression of *hsa_circ_0001947* among newly diagnosed, under-treatment, and relapsed AML patients indicates its potential as a biomarker for disease progression and response to therapy.

These findings suggest that *hsa_circ_0001947* may influence AML development through interactions with the TGF-β signaling pathway, possibly affecting cell proliferation, apoptosis, and immune evasion. Further functional studies are needed to elucidate its precise molecular mechanisms and explore its therapeutic potential in AML management.

Conflict of interest: The authors declare that there are no conflicts of interest related to this work.

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