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ORIGINAL RESEARCH

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Diagnostic and prognostic significance of internal tRNA-derived fragment glycine-glycine-cytosine (itRNA^{GlyGCC}) expression in epithelial ovarian cancer

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Abstract

Background: Epithelial ovarian cancer (EOC) is a leading cause of cancer death in women, often diagnosed at advanced stages due to its vague early symptoms. This study investigates the potential of a small non-coding RNA fragment, internal tRNA-derived fragment Glycine-Glycine-Cytosine (i-tRF^{GlyGCC}), as a biomarker for EOC diagnosis and prognosis. **Methods**: The study involved 142 patients with stage III EOC and 100 healthy controls. i-tRF^{GlyGCC} expression was measured in tumor tissues, serum samples, and analyzed using real-time polymerase chain reaction (PCR) . Receiver operating characteristic (ROC) curves assessed diagnostic accuracy. Correlations between i-tRF^{GlyGCC} expression and clinicopathological features, treatment response, and survival outcomes were investigated. **Results**: We observed significantly lower i-tRF^{GlyGCC} expression in both EOC tissues and serum compared to controls. Notably, ROC curve analysis revealed exceptional diagnostic accuracy for i-tRF^{GlyGCC} in both tissues (Area Under the Curve (AUC) = 1.000) and serum (AUC = 1.000), suggesting its potential as a diagnostic tool. Furthermore, we explored the relationship between i-tRF^{GlyGCC} expression and various EOC prognostic features. We found a strong negative correlation with patient age and significant associations with advanced stage, non-serous tumor type, poorer tumor differentiation, presence of ascites, lower treatment response, and larger residual tumor burden. Additionally, patients with high i-tRF^{GlyGCC} expression exhibited significantly poorer overall and disease-free survival rates. **Conclusions**: This study highlights the promise of i-tRF^{GlyGCC} as a biomarker for EOC diagnosis and prognosis. Its downregulation in EOC coupled with its correlation with aggressive features and poorer survival outcomes warrants further investigation in larger studies to validate its clinical utility.

Keywords

Epithelial ovarian cancer; Internal tRNA-derived fragment Glycine-CCC; Diagnostic marker; Prognostic marker; Survival

1. Introduction

Epithelial ovarian cancer (EOC) was estimated to account for 4.7% of cancer deaths and 3.7% of cases worldwide, making it the eighth most prevalent cancer in women [1]. The two primary characteristics of EOC are its insidious progression and late-stage diagnosis; early and accurate identification of the disease is crucial for improving survival rates [2]. Approximately 70% of cases of EOC are diagnosed at an advanced stage, resulting in a 5-year survival rate of only 30% for patients. However, if EOC is identified early and confined to the ovaries, the 5-year survival rate could be as high as 90% [3].

The early symptoms of EOC are vague, making it challenging to identify the disease early despite advancements in our understanding of its molecular and cellular basis. This highlights the urgent need for new molecular biomarker-based therapeutic approaches and new diagnostic and prognostic markers.

Among the diverse types of non-coding RNAs (ncRNAs), tRNA-derived RNA fragments (tRFs) have recently emerged as a promising area with significant diagnostic and prognostic potential [4]. These endogenous, single-stranded non-coding RNA fragments typically range from 14 to 40 nucleotides in length and exhibit functional similarities to other ncRNA classes, such as miRNAs, playing a critical role in the regulation of gene expression [5, 6]. Despite ongoing research, the precise biological functions of transfer RNAs

(tRNAs) remain incompletely understood. One notable example is the internal tRNA-derived fragment GlyGCC (i-tRF^{GlyGCC}), which has the nucleotide sequence 5′-GCATTGGTGGTTCAGTGGTAGAATTCTCGC-3′. This 30-nucleotide fragment is derived from the internal regions of mature glycine tRNA containing the GCC anticodon and is generated through specific enzymatic cleavage of the parent tRNA [7].

Emerging evidence suggests that i-tRF^{GlyGCC} is not merely a byproduct of tRNA degradation but a functionally significant molecule with diverse biological roles. Dysregulation of i-tRF^{GlyGCC} expression has been observed in various pathological conditions, particularly in cancer development and progression [8]. Mechanistically, i-tRF^{GlyGCC} has been implicated in interactions with key cellular proteins and the regulation of gene expression through both transcriptional and post-transcriptional mechanisms [9]. Studies have demonstrated its involvement in critical cellular processes, including cell proliferation, apoptosis and stress response pathways [10]. For instance, Yang *et al.* [11] reported that i-tRF^{GlyGCC} can modulate protein synthesis by competing with full-length tRNAs. At the same time, Sarkar *et al.* [12] identified its role in microRNA-like gene silencing through direct interactions with target mRNAs.

The evolutionary conservation of i-tRF^{GlyGCC} across species, combined with its tissue-specific expression patterns, underscores its fundamental role in cellular regulation. This molecular signature has emerged as a crucial factor in both physiological and pathological processes, particularly in cancer biology [13]. tRNAs demonstrate a dual regulatory function in malignancy, acting as both oncogenic and oncostatic elements through their roles as inducers and blockers of cancer-related pathways [9]. Their tumor-suppressive effects are mediated through post-transcriptional regulation of cancer-associated genes, operating via transcriptional control mechanisms and malignant gene stabilization [14].

Studies have expanded our understanding of tRF involvement in disease pathogenesis. These molecules have been implicated in a broad spectrum of pathological conditions, including hematologic malignancies [15], solid tumors [16], metabolic disorders [17], inflammatory conditions [13], viral infections [18] and neurological diseases [9]. This diverse disease association highlights the expansive regulatory network influenced by tRF expression and function. Of particular significance is tRF-03357, which has demonstrated the capacity to enhance epithelial ovarian cancer (EOC) cell migration, invasion, and proliferation *in vitro* [19]. Furthermore, i-tRFs derived from tRNA-Gly have emerged as promising diagnostic biomarkers for ovarian cancer, exhibiting distinctive expression patterns in patient serum compared to healthy controls [20]. The high specificity and sensitivity of these i-tRFs in ovarian cancer prediction, coupled with their dysregulation in malignancy and prevalence in body fluids, position them as valuable potential molecular markers for EOC diagnosis and monitoring.

This study investigates the potential of i-tRF^{GlyGCC} as a diagnostic and prognostic molecular marker in EOC.

2. Patients and methods

The study involved 142 patients diagnosed with stage III epithelial ovarian cancer according to the International Federation of Gynecology and Obstetrics (FIGO) staging, between May 2018 and June 2024 [21]. Pathological examination confirmed epithelial ovarian cancer (EOC). Debulking surgery had been performed on each patient within two months before the first course of chemotherapy. One hundred healthy women volunteered to serve as controls for the study.

The inclusions included being 18 or older, having normal baseline blood counts, and demonstrating normal kidney and liver function.

The study excluded those with ovarian low-malignant tumors, a performance status exceeding two on the Eastern Cooperative Oncology Group (ECOG) scale [22], or a glomerular filtration rate (GFR) below 60 mL/minute. Additionally, patients with severe neuropathy, a history of prior chemotherapy or radiotherapy for ovarian cancer, congestive heart failure, or arrhythmias were omitted.

All participants provided written informed consent after Zagazig University's Research Ethics Committee granted authorization (ZU-232/2018).

After surgery, all patients received a standardized postoperative chemotherapy regimen. This regimen included six cycles; each repeated every three weeks. Each cycle consisted of the following treatments:

- Paclitaxel: administered intravenously at a 175 mg/m^2 dose over three hours.
- Cisplatin: administered intravenously at a 75 mg/m^2 dose following adequate patient hydration.

2.1 Patient monitoring

Patients underwent regular evaluations throughout the study to monitor treatment response and potential side effects. These evaluations included gynecological examinations to assess overall health and identify any gynecological abnormalities. Abdominopelvic ultrasonography was used to visualize the pelvic organs and surrounding tissues for signs of recurrence.

Serum levels of the cancer antigen 125 (CA-125) were also measured using CA-125 assays. Baseline and follow-up computed tomography (CT) or magnetic resonance imaging (MRI) scans of the abdomen and pelvis were performed before starting chemotherapy and repeated every two months. The revised Response Evaluation Criteria in Solid Tumors (RECIST) criteria were used to assess tumor response based on these scans [16].

2.2 Post-treatment assessment

After completing the six chemotherapy cycles, patients who achieved a complete clinical response underwent laparoscopy. If laparoscopy revealed no signs of cancer, a laparotomy was performed for exploration, and multiple tissue biopsies were taken for pathological evaluation.

Based on the microscopic examination of these biopsies, patients were categorized into three groups:

1. Complete Response (CR): No evidence of cancer cells was found.

2. Partial Response (PR): There was an identification of microscopic evidence of residual cancer cells, indicating minimal disease.

3. Persistent Disease: There was macroscopic or microscopic evidence of remaining cancer.

Tissue samples were collected from 142 women who underwent surgery for epithelial ovarian cancer at Zagazig University Hospitals. The samples were taken from both the tumor and the adjacent normal ovarian tissues (ANOT). A pathologist examined each tissue sample histologically before quickly freezing it in liquid nitrogen.

2.3 Total RNA extraction

Tissue and blood samples were collected from 142 women who underwent surgery for epithelial ovarian cancer at Zagazig University Hospitals. Blood samples were also collected from 100 age-matched healthy controls. Tissue samples were taken from both the tumor and ANOT during surgery. A pathologist examined each tissue sample histologically. All tissue and serum samples were frozen in liquid nitrogen at −196 °C.

We extracted total RNA using TRI-Reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA, catalog number 15596026) from 40–150 mg of homogenized tissue samples and 250 μL of serum samples following the manufacturer's instructions. The extracted RNA was dissolved in RNA Storage Solution (Thermo Fisher Scientific Inc., Waltham, MA, USA, catalog number AM7001). To ensure the quality of the RNA, we performed spectrophotometric measurements at 260 and 280 nm and calculated the absorbance ratios at 260/280 nm and 260/230 nm. Additionally, we assessed the RNA quality using agarose gel electrophoresis, providing solid evidence for the reliability of our results.

2.4 Polyadenylation of RNA and synthesis of the first cDNA strand

A polyadenylation step was performed to prepare the total RNA for reverse transcription. This involved adding 1 μg of total RNA to a reaction mixture containing 800 μM ATP and 1 U of E. coli poly(A) polymerase (Thermo Fisher Scientific Inc., Waltham, MA, USA). The reaction was incubated at 37 °C for 60 minutes to allow polyadenylation of the RNA's 3′-end. Polymerase inactivation was achieved by heating the reaction mixture to 65 °C for 15 minutes.

Following polyadenylation, the first-strand cDNA synthesis was carried out using 50 U of MMLV reverse transcriptase (Thermo Fisher Scientific Inc., Waltham, MA, USA), 40 U of recombinant ribonuclease inhibitor (Invitrogen), and 0.25 μM of the oligo(dT) adapter. The oligo(dT) adapter has the following structure: 5′-GCGAGCACAGAATTAATACGACTCACTATAGGTTTTT TTTTTTTVN-3′, where V represents the degenerate bases G, A, or C and N represents the degenerate bases G, A, T or C. The reaction mixture, containing the polyadenylated RNA, was incubated at 37 °C for 60 minutes to allow reverse transcription. Finally, the MMLV reverse transcriptase was inactivated by heating the reaction mixture to 70 °C for 15 minutes.

2.5 Quantitative real-time polymerase chain reaction (qPCR)

For the measurement of i-tRF^{GlyGCC} levels, we used quantitative real-time PCR qPCR techniques with SYBR-Green fluorescence [23]. To ensure accurate results, we utilized the short nucleolar RNA C/D box 48 (SNORD48, also known as RNU48) as an endogenous reference control for normalization due to its stable expression across different tissue types. We designed specific forward primers for SNORD48 (5′-TGATGATGACCCCAGGTAACTCT-3′) and i-tRF^{GlyGCC} (5′-GAGGCCCGGGTTCGATTC-3′) based on their known sequences. These forward primers were paired with a universal reverse primer (5′-GCGAGCACAGAATTAATACGAC-3′) to generate specific amplicons. We used 2 ng of cDNA and 150 nM of each qPCR primer for the tests. The levels of the small non-coding RNA in each sample were determined using the comparative Ct ($2^{-\Delta\Delta Ct}$) method.

2.6 Statistical analysis

Statistical analysis was conducted using IBM SPSS Statistics 27 (IBM Corp., Armonk, NY, USA). The data are presented as mean ± standard deviation (SD), with statistical significance set at $p < 0.05$. For paired data with a normal distribution, paired *t*-tests were employed to compare differences between groups. One-way analysis of variance (ANOVA) was used to assess differences among three or more groups. Receiver operating characteristic (ROC) curves were generated to evaluate the diagnostic efficiency of the biomarkers, and the area under the curve (AUC), specificity and sensitivity were calculated to assess their discriminatory power. Survival analysis was performed using Kaplan-Meier curves, which estimate the probability of survival over time, and differences between survival curves were evaluated using the log-rank test to determine statistical significance.

3. Results

3.1 Patient clinical characteristics

A total of 146 patients participated in the study, ranging in age from 18 to 66 years, with a median age of 41.5. The distribution of cancer stages was as follows: 33 patients (22.6%) with stage IIIA, 30 patients (20.5%) with stage IIIB, and 83 patients (56.8%) with stage IIIC. Regarding histopathology, 91 patients (62.3%) had serous carcinoma, while 55 patients (37.7%) had non-serous carcinoma. In addition, 87 patients (59.6%) had poorly differentiated tumors.

Ascites was present in 89 patients (61.0%). After the initial debulking surgery, 89 patients (61.0%) had residual tumors less than 2 cm in size, whereas 57 patients (39.0%) had residual tumors larger than 2 cm. Regarding the chemotherapy response, 75 patients (51.4%) exhibited either a complete response, residual microscopic disease or a partial response.

3.2 Expression of i-tRF^{GlyGCC} in epithelial ovarian cancer: a potential biomarker?

The expression of i-tRF^{GlyGCC} was found to be downregulated in epithelial ovarian cancer. In epithelial ovarian cancer samples, the mean ± SD level of i-tRF^{GlyGCC} was 5.995 ± 3.302. In contrast, in normal ovarian tissue (ANOT), it was 0.4080 ± 0.1707, indicating a statistically significant difference ($p < 0.0001$) (Fig. 1A). Similar results were observed in the serum of patients with epithelial ovarian cancer compared to healthy controls. The mean expression of i-tRF^{GlyGCC} in the serum of ovarian cancer patients was 6.579 ± 2.949, while in the control group, it was 0.3666 ± 0.1905, again demonstrating a statistically significant difference ($p < 0.0001$) (Fig. 1B).

The effectiveness of i-tRF^{GlyGCC} as a diagnostic marker for epithelial ovarian cancer (EOC) was evaluated by analyzing its performance using receiver operating characteristic (ROC) curves. The findings revealed exceptional diagnostic accuracy for i-tRF^{GlyGCC} in EOC tissues (Fig. 1C) and patient serum samples (Fig. 1D). In EOC tissues, i-tRF^{GlyGCC} perfectly differentiated between malignant and normal ovarian tissues, with an AUC of 1.000 (95% Confidence Interval (CI): 0.9998–1.000; $p < 0.0001$). Similarly impressive results were observed in the serum analysis, where i-tRF^{GlyGCC} effectively distinguished EOC patients from controls (AUC = 1.000; 95% CI: 1.000–1.000; $p < 0.0001$).

We studied the relationship between i-tRF^{GlyGCC} levels in epithelial ovarian cancer (EOC) tissues and patient serum. The results revealed a robust positive correlation, indicating that tissue and serum levels closely mirror each other ($R^2 = 0.9955$, 95% CI: 0.9969–0.9984, $p < 0.0001$). In simpler terms, as the level of i-tRF^{GlyGCC} increases in tissue samples, the level in serum samples also tends to increase proportionally. This strong correlation suggests that i-tRF^{GlyGCC} in serum might be a promising non-invasive biomarker for EOC (Fig. 2).

3.3 Prognostic significance of i-tRF^{GlyGCC} expression in epithelial ovarian cancer

In this study, we investigated the potential of i-tRF^{GlyGCC} as a prognostic biomarker for epithelial ovarian cancer (EOC) patients. Interestingly, we found a strong negative correlation between patient age and i-tRF^{GlyGCC} expression ($r = -0.9911$). In simpler terms, as patients' age increased, their i-tRF^{GlyGCC} levels significantly decreased (p-value < 0.0001) (Fig. 3).

Our data indicates a strong correlation between i-tRF^{GlyGCC} expression and several prognostic features in EOC patients (Table 1).

Stage: Patients with higher stages (IIIB & IIIC) have significantly higher i-tRF^{GlyGCC} levels compared to Stage IIIA (p-value < 0.0001). This suggests a potential link between the advanced disease stage and increased i-tRF^{GlyGCC} expression.

Pathology: Serous tumors, the most common type of EOC, have lower i-tRF^{GlyGCC} levels than non-serous tumors (p-value < 0.0001). This indicates a possible association between tumor type and i-tRF^{GlyGCC} expression.

Grade: Patients with poorly differentiated tumors (more aggressive) have significantly higher i-tRF^{GlyGCC} levels compared to those with moderately or well-differentiated tumors (p-value < 0.0001). This suggests a potential association between higher tumor grade and increased i-tRF^{GlyGCC} expression.

Ascites: Patients with ascites (fluid buildup in the abdomen) have significantly higher i-tRF^{GlyGCC} levels compared to those without ascites (p-value < 0.0001). This suggests a possible correlation between ascites and increased i-tRF^{GlyGCC} expression.

Response to Treatment: Patients with complete response (CR), microscopic disease, or partial response (PR) have significantly lower i-tRF^{GlyGCC} levels compared to those with no response or progressive disease (p-value < 0.0001). This suggests a potential link between better treatment response and lower i-tRF^{GlyGCC} expression.

Residual Disease: Patients with minimal residual disease (<2 cm) have significantly lower i-tRF^{GlyGCC} levels compared to those with larger residual tumors (>2 cm) (p-value < 0.0001). This suggests a possible association between lower residual disease burden and decreased i-tRF^{GlyGCC} expression.

These results highlight a promising link between i-tRF^{GlyGCC} expression and various prognostic features in EOC. Higher i-tRF^{GlyGCC} levels are associated with advanced stage, aggressive tumor characteristics, and poorer treatment outcomes.

In the multiple regression analysis, we examined how the dependent variable A (EOC) relates to a set of independent variables. The model included ten independent variables: B (ANOT), C (Stage), D (Age), E (serum), F (Pathology), G (Grade), H (Ascites), I (Response) and J (Residual Disease). After examining the parameter estimates, we found that only three variables, C (Stage), F (Pathology) and I (Response), showed statistically significant relationships with the dependent variable. These variables had p-values below the threshold of 0.05, indicating a significant association (Table 2).

3.4 i-tRF^{GlyGCC} expression and its association with survival outcomes in EOC patients

High expression of i-tRF^{GlyGCC} is linked to poorer overall and disease-free survival in patients with EOC. Patients with high i-tRF^{GlyGCC} expression had a median overall survival of 36 months, while those with low expression had 61 months ($p = 0.0032$), indicating a 25-month difference in median survival time. Likewise, the median disease-free survival was 42 months for high-expression patients and 61 months for low-expression patients ($p = 0.0128$), suggesting that patients with high i-tRF^{GlyGCC} expression experienced disease recurrence on average 19 months earlier (Fig. 4).

These findings suggest that i-tRF^{GlyGCC} expression could serve as a prognostic marker for EOC survival.

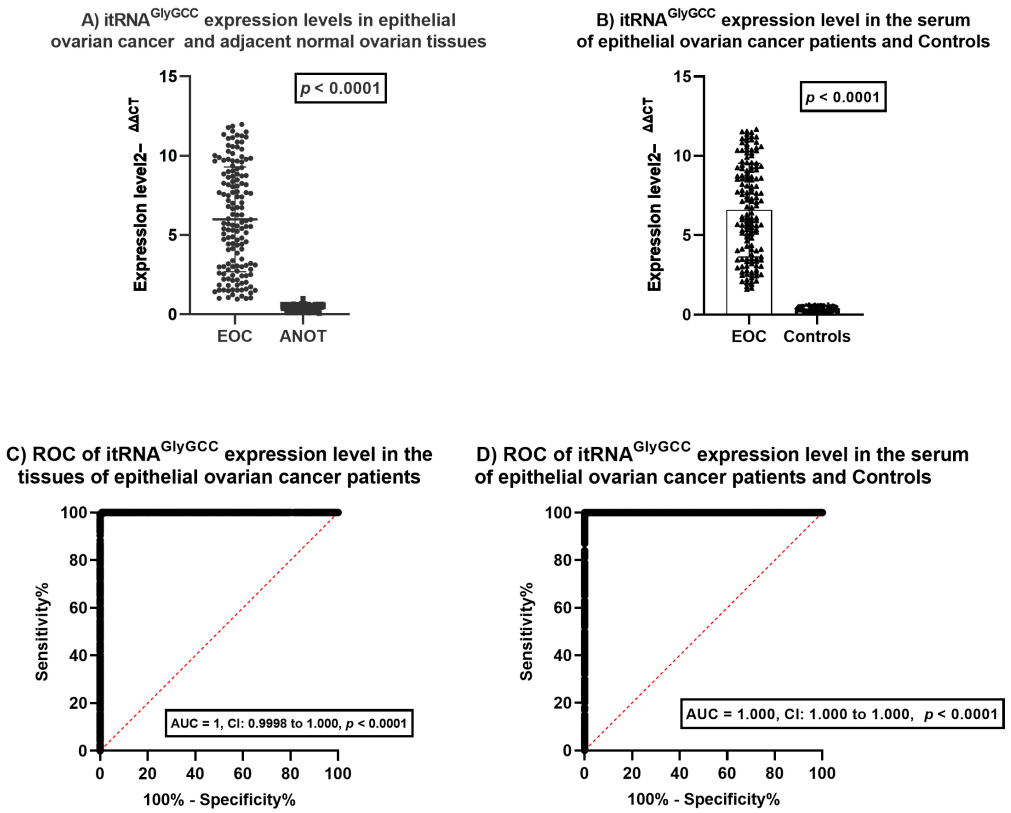


FIGURE 1. Expression and diagnostic performance of i-tRF^{GlyGCC} in epithelial ovarian cancer (EOC). (A) i-tRF^{GlyGCC} expression levels in EOC tissues compared to adjacent normal ovarian tissues (ANOT). (B) i-tRF^{GlyGCC} expression levels in the serum of EOC patients versus healthy controls. (C) Receiver operating characteristic (ROC) curve analysis of i-tRF^{GlyGCC} expression in EOC tissues, demonstrating diagnostic accuracy (AUC = 1.000). (D) ROC curve analysis of i-tRF^{GlyGCC} expression in serum, showing diagnostic accuracy (AUC = 1.000). All comparisons were statistically significant ($p < 0.0001$). AUC: Area Under Curve; i-tRF^{GlyGCC}: internal tRNA-derived fragment Glycine-Glycine-Cytosine; CI: Confidence interval.

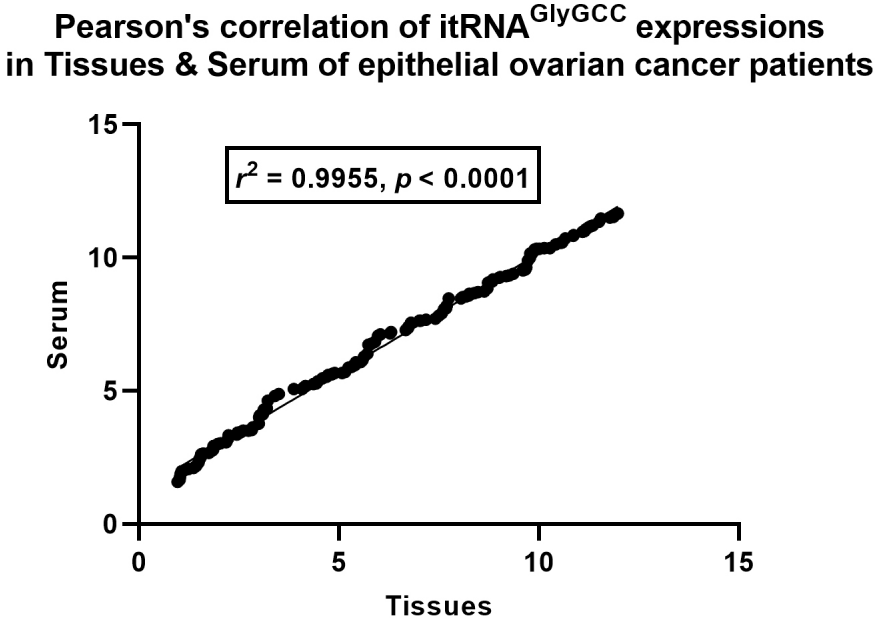


FIGURE 2. Correlation of i-tRF^{GlyGCC} expression in tissues and serum of epithelial ovarian cancer (EOC) patients. Pearson's correlation analysis demonstrates a strong positive correlation ($r^2 = 0.9955$, $p < 0.0001$) between i-tRF^{GlyGCC} expression levels in tumor tissues and serum samples. i-tRF^{GlyGCC}: internal tRNA-derived fragment Glycine-Glycine-Cytosine.

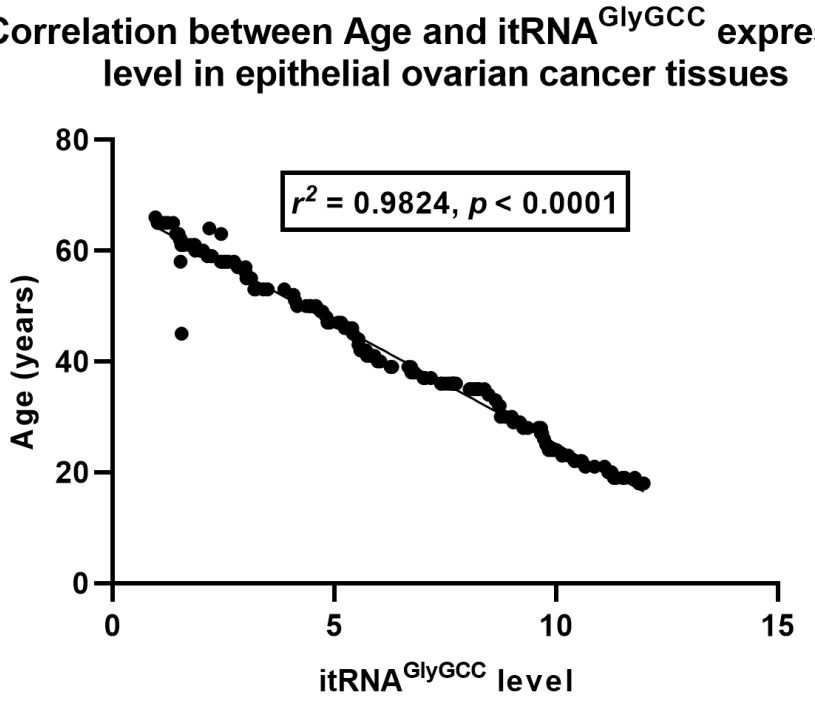


FIGURE 3. Correlation between age and i-tRF^{GlyGCC} expression in epithelial ovarian cancer (EOC) tissues. Pearson's correlation analysis reveals a strong positive correlation ($r = 0.9824$, $p < 0.0001$) between patient age and i-tRF^{GlyGCC} expression levels in EOC tissues. i-tRF^{GlyGCC}: internal tRNA-derived fragment Glycine-Glycine-Cytosine.

TABLE 1. itRNA^{GlyGCC} expression in epithelial ovarian cancer according to different clinical and pathological features.

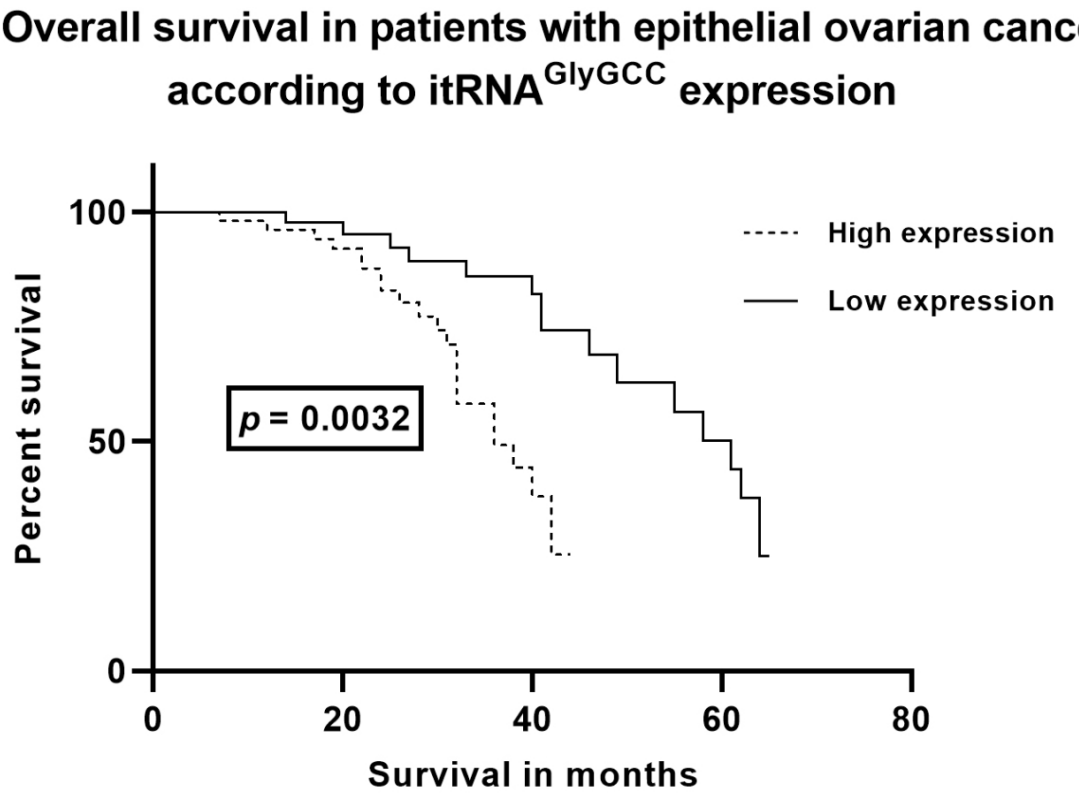
	No	itRNA^{GlyGCC}		*t*-test	*p*
		Mean	SD		
Stage					
IIIA	33	2.523	2.37		
IIIB	30	3.613	0.94	118.20*	<0.0001
IIIC	83	8.236	2.18		
Pathology					
Serous	91	4.078	2.28	4.97	<0.0001
Non-serous	55	6.466	3.97		
Grade					
Moderate + Well differentiated	59	2.602	1.14	19.30	<0.0001
Poorly differentiated	87	8.296	2.06		
Ascites					
Absent	57	2.525	1.09	18.87	<0.0001
Present	89	8.217	2.10		
Response					
CR, Microscopic disease, PR	75	3.197	1.54	21.59	<0.0001
No response or progressive disease	71	8.951	1.68		
Residual Disease					
>2 cm	57	8.217	2.10	18.87	<0.0001
<2 cm	89	2.525	1.09		

**ANOVA test. itRNA^{GlyGCC}: internal tRNA-derived fragment Glycine-Glycine-Cytosine; SD: Stable Disease; CR: Complete Response; PR: Partial Response.*

TABLE 2. Multiple regression analysis of different prognostic features.

Regression Statistics								
Multiple *R*	1							
R Square	1							
Adjusted R Square	0.992701							
Standard Error	1.65×10^{-15}							
Observations	146							
	ANOVA							
	df	SS	MS	*F*	Significance *F*			
Regression	9	1580.858	175.6509	7.28328×10^{31}	0			
Residual	137	3.72×10^{-28}	2.71×10^{-30}					
Total	146	1580.858						
	Coefficients	Standard Error	*t* Stat	*p*-value	Lower 95%	Upper 95%	Lower 95.0%	Upper 95.0%
Intercept	-6.9×10^{-15}	1.05×10^{-15}	−6.53821	1.13763×10^{-9}	-8.9×10^{-15}	-4.8×10^{-15}	-8.9×10^{-15}	-4.8×10^{-15}
ANOT	-3×10^{-17}	8.16×10^{-16}	−0.0369	0.97061942	-1.6×10^{-15}	1.58×10^{-15}	-1.6×10^{-15}	1.58×10^{-15}
Stage	1.79×10^{-16}	6.7×10^{-17}	2.668292	0.008543895	4.63×10^{-17}	3.11×10^{-16}	4.63×10^{-17}	3.11×10^{-16}
Age	1	5.01×10^{-16}	1.99×10^{15}	0	1	1	1	1
Serum	-2.7×10^{-16}	5.49×10^{-16}	−0.49673	0.620173186	-1.4×10^{-15}	8.13×10^{-16}	-1.4×10^{-15}	8.13×10^{-16}
Pathology	2.23×10^{-16}	4.91×10^{-17}	4.554181	1.15064×10^{-5}	1.26×10^{-16}	3.2×10^{-16}	1.26×10^{-16}	3.2×10^{-16}
Grade	2.87×10^{-17}	1.23×10^{-16}	0.232542	0.816464479	-2.2×10^{-16}	2.73×10^{-16}	-2.2×10^{-16}	2.73×10^{-16}
Ascites	2.02×10^{-17}	1.13×10^{-16}	0.178279	0.858767184	-2×10^{-16}	2.44×10^{-16}	-2×10^{-16}	2.44×10^{-16}
Response	1.36×10^{-16}	5.4×10^{-17}	2.509885	0.013240946	2.88×10^{-17}	2.43×10^{-16}	2.88×10^{-17}	2.43×10^{-16}
Residual Disease	0	0	65,535	0.817564432	0	0	0	0

ANOVA: Analysis of Variance; df: the degrees of freedom; SS: the sum of squares; MS: Mean Squared Errors; ANOT: Adjacent normal ovarian tissues.



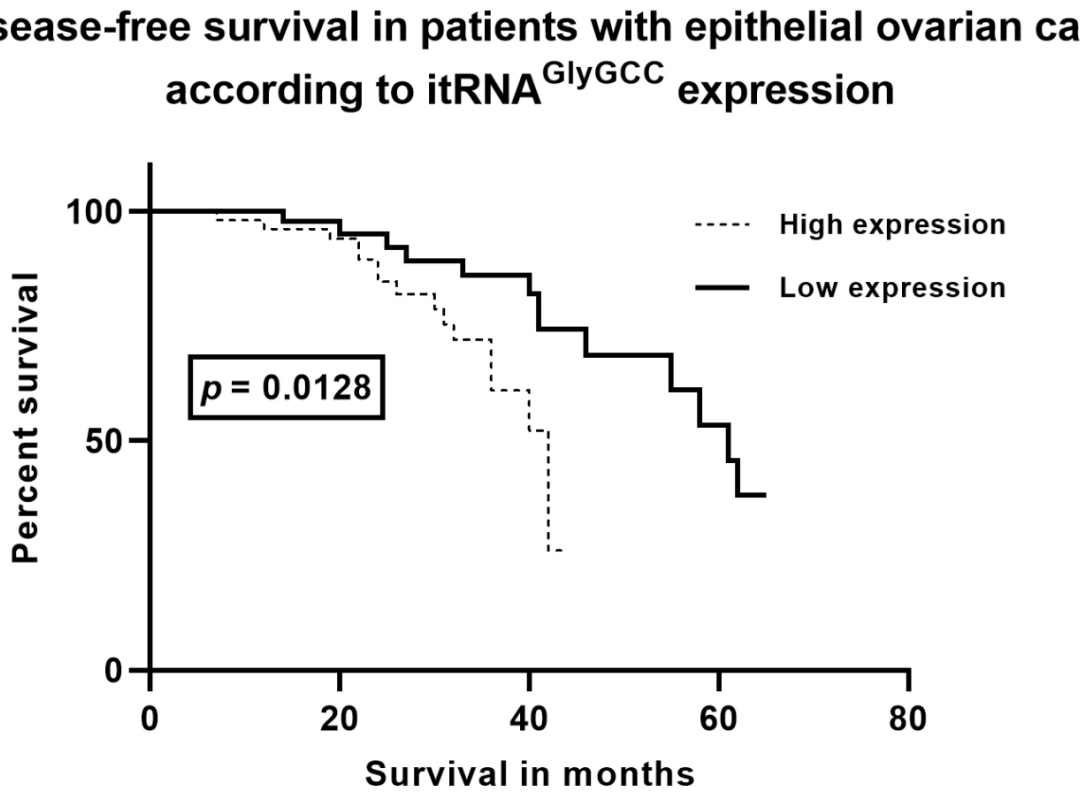


FIGURE 4. Survival analysis based on i-tRF^{GlyGCC} expression in epithelial ovarian cancer (EOC) patients. (A) Overall survival (OS) in EOC patients stratified by high and low i-tRF^{GlyGCC} expression levels (p = 0.0032). (B) Disease-free survival (DFS) in EOC patients stratified by high and low i-tRF^{GlyGCC} expression levels (p = 0.0128). High i-tRF^{GlyGCC} expression is associated with significantly poorer survival outcomes. i-tRF^{GlyGCC}: internal tRNA-derived fragment Glycine-Glycine-Cytosine.

4. Discussion

Ovarian cancer is a highly lethal malignancy, primarily due to the challenges associated with its early diagnosis [24]. In its initial stages, the disease often presents with nonspecific symptoms, such as bloating, pelvic pain and changes in bowel habits, which are easily mistaken for benign conditions [25]. This lack of distinct clinical manifestations frequently results in delayed or misdiagnosis, significantly worsening patient prognosis [26]. Furthermore, the absence of reliable and accessible screening methods exacerbates the difficulty of early detection. Unlike breast or cervical cancer, for which routine screening tests are well-established, ovarian cancer lacks widely available diagnostic tools, allowing the disease to progress undetected until advanced stages [26]. This underscores the critical need for the development of effective early detection strategies to improve outcomes for patients with ovarian cancer.

Recent research has highlighted the potential of i-tRF^{GlyGCC}, a transfer RNA (tRNA)-derived fragment, as a promising biomarker in various cancers. Studies have shown significantly elevated levels of i-tRF^{GlyGCC} in the blood plasma of colorectal cancer (CRC) patients compared to healthy individuals, suggesting its potential utility in developing non-invasive blood-based diagnostic tests for CRC [27]. Additionally, research has identified a positive correlation between increased i-tRF^{GlyGCC} expression and poorer overall and disease-free survival rates in patients with chronic lymphocytic leukemia (CLL), underscoring its prognostic value [28]. These findings indicate that i-tRF^{GlyGCC} could serve as a valuable tool for early cancer detection, risk stratification, and the development of personalized treatment strategies, offering new avenues for improving cancer management and patient outcomes.

In this study, we investigated the expression levels of i-tRF^{GlyGCC} in both tumor tissues and serum samples from epithelial ovarian cancer (EOC) patients to evaluate its potential as a diagnostic and prognostic biomarker.

The expression of i-tRF^{GlyGCC} was significantly downregulated in epithelial ovarian cancer (EOC) tissues compared to normal ovarian tissues, with a statistically significant difference ($p < 0.0001$). Consistent findings were observed in serum samples, where i-tRF^{GlyGCC} levels were significantly lower in ovarian cancer patients compared to healthy controls, further demonstrating a statistically significant difference ($p < 0.0001$).

Our study explored the relationship between i-tRF^{GlyGCC} levels in epithelial ovarian cancer (EOC) tissues and corresponding serum samples. Notably, we identified a strong positive correlation between i-tRF^{GlyGCC} expression in tissues and serum, indicating that elevated levels in tissue samples were associated with similarly increased levels in the blood. This close correlation suggests that measuring i-tRF^{GlyGCC} in serum could serve as a reliable, non-invasive method for diagnosing EOC. These findings underscore the potential of serum i-tRF^{GlyGCC} as a promising non-invasive biomarker for epithelial ovarian cancer, offering a practical alternative to tissue biopsies, which are more invasive and challenging to obtain.

A study by Christodoulou *et al.* [7] investigated the prognostic significance of the tRNA fragment i-tRF^{GlyGCC} in colorectal cancer (CRC) patients. By analyzing total RNA from 91 cancerous tissue samples and 83 adjacent normal tissues, the study revealed significantly reduced levels of i-tRF^{GlyGCC} in CRC tissues compared to normal colorectal tissues. Similarly, another study explored the potential of plasma exosomal tRNA-derived fragments (tRFs) as diagnostic biomarkers for non-small cell lung cancer (NSCLC). The study identified a specific panel of tRFs, including tRF-Leu-TAA-005, tRF-Asn-GTT-010, tRF-Ala-AGC-036, tRF-Lys-CTT-049 and tRF-Trp-CCA-057, which were significantly downregulated in NSCLC patients compared to healthy individuals [29]. Additionally, research on bladder cancer examined tRFs in urine and blood extracellular vesicles (EVs) as noninvasive biomarkers. Distinct tRF profiles were observed in urine EVs from cancer patients compared to healthy individuals, and serum tRF levels changed following surgery, highlighting their potential for bladder cancer diagnosis and monitoring [30]. Furthermore, Li *et al.* [31] studied tRF-Pro-CGG in pancreatic ductal adenocarcinoma (PDAC) and found significantly lower levels of tRF-Pro-CGG in PDAC patients, underscoring its potential role in this malignancy. Collectively, these studies demonstrate the growing interest in tRFs as promising diagnostic and prognostic biomarkers across various cancer types.

The current study identified a strong correlation between i-tRF^{GlyGCC} expression and several indicators of poor prognosis in epithelial ovarian cancer (EOC). Patients with advanced-stage disease, non-serous histology, higher tumor grades, ascites, poorer treatment response, and larger residual tumors exhibited significantly elevated levels of i-tRF^{GlyGCC} ($p < 0.0001$). These findings suggest that i-tRF^{GlyGCC} may serve as a potential biomarker for aggressive EOC and unfavorable clinical outcomes. Similarly, in pancreatic ductal adenocarcinoma (PDAC), Li *et al.* [31] reported that lower levels of tRF-Pro-CGG were associated with poorer prognosis and reduced survival, highlighting its potential as a biomarker for disease progression and treatment guidance. In gastric cancer, Gan *et al.* [32] demonstrated that dysregulation of tsRNAs was closely linked to adverse clinicopathological factors, including lymph node metastasis, advanced Tumor-Node-Metastasis (TNM) stage, larger tumor size, and vascular invasion. Additionally, Zhang *et al.* [33] identified three specific tRNA fragments (tRFs)—tRF-Gly-CCC-046, tRF-Tyr-GTA-010 and tRF-Pro-TGG-001—that were significantly downregulated in both tissue and blood samples from breast cancer patients compared to healthy individuals, suggesting their potential as biomarkers for early breast cancer detection. Collectively, these studies underscore the emerging role of tRNA-derived fragments as valuable biomarkers for cancer diagnosis, prognosis, and therapeutic decision-making across various malignancies.

In our study, we observed that epithelial ovarian cancer (EOC) patients with elevated levels of i-tRF^{GlyGCC} faced a significantly worse prognosis. Specifically, these patients had a median overall survival (OS) that was 25 months shorter (36 months *vs.* 61 months) and experienced disease recurrence 19 months earlier compared to those with lower i-tRF^{GlyGCC} levels. Similarly, in colorectal cancer (CRC), patients with high

i-tRF^{GlyGCC} levels exhibited significantly poorer outcomes, including shorter disease-free intervals (DFS) and overall survival (OS), compared to those with lower levels [7]. Consistent with these findings, Karousi *et al.* [18] reported that increased levels of i-tRF^{GlyGCC} were associated with poor overall survival in chronic lymphocytic leukemia (CLL) patients. These results collectively highlight the prognostic significance of i-tRF^{GlyGCC} across multiple cancer types, underscoring its potential as a biomarker for predicting aggressive disease and unfavorable clinical outcomes.

This study identifies i-tRF^{GlyGCC} as a promising biomarker for the diagnosis and prognosis of epithelial ovarian cancer (EOC). However, further research is required to validate and confirm these findings, ensuring their reproducibility and clinical applicability.

5. Limitations of the study

Our study has several limitations, including a relatively small and homogeneous sample size of 142 stage III epithelial ovarian cancer patients, which may not fully represent the broader population or earlier disease stages. Conducted at a single institution (Zagazig University Hospitals), the findings may lack generalizability due to potential institutional biases and limited geographic or ethnic diversity. The study also lacked long-term follow-up data, which could provide deeper insights into the prognostic value of i-tRF^{GlyGCC} over time. Additionally, potential confounding factors such as comorbidities, lifestyle, and genetic predispositions were not accounted for, and the reliance on qPCR for measuring i-tRF^{GlyGCC} levels may introduce technical variability. As an exploratory study, the findings require validation in larger, multicenter cohorts, and the absence of functional studies limits the understanding of the biological mechanisms underlying i-tRF^{GlyGCC}'s role in ovarian cancer progression. These limitations underscore the need for further research to confirm and expand upon these results.

6. Conclusions

In conclusion, this study presents compelling evidence for i-tRF^{GlyGCC} as a promising novel biomarker in epithelial ovarian cancer. Our findings demonstrate significantly reduced i-tRF^{GlyGCC} expression in both tumor tissues and serum of EOC patients compared to healthy controls, with perfect diagnostic accuracy as indicated by ROC curve analysis. The strong associations between i-tRF^{GlyGCC} expression and adverse clinicopathological features, including advanced stage, poor differentiation, and presence of ascites, alongside its correlation with diminished survival outcomes, suggest its potential value as both a diagnostic and prognostic marker. These results provide a strong foundation for the clinical application of i-tRF^{GlyGCC} in EOC management, though larger multicenter studies are needed to validate these findings and establish standardized cutoff values for clinical implementation. Our study contributes to the growing understanding of tRNA-derived fragments in cancer biology and opens new avenues for improving EOC patient care through enhanced early detection and more accurate prognostic assessment.

AVAILABILITY OF DATA AND MATERIALS

All data will be available upon request.

AUTHOR CONTRIBUTIONS

HD, AFG, WHE—Conceptualization, Methodology, Software. AAA, AA, MMB, RAK—Data curation, Writing-Original draft preparation. AAA, AFG, WHE—Supervision. AAA—Software, Validation. TMA, AFG, WHE—Writing-Reviewing and Editing. All authors have read the manuscript and approved the submission.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All participants provided written informed consent after Zagazig University's Research Ethics Committee granted authorization (ZU-232/2018).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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