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Research paper

Identification of a novel, internal tRNA-derived RNA fragment as a new prognostic and screening biomarker in chronic lymphocytic leukemia, using an innovative quantitative real-time PCR assay

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ABSTRACT

Chronic lymphocytic leukemia (CLL) is one of the most common types of leukemia in adults. Several studies have identified various prognostic biomarkers in CLL. In this study, we investigated the potential value of an internal fragment of the tRNAs bearing the Glycine anticodon CCC (i-tRF-GlyCCC), which is a small non-coding RNA, as a prognostic and screening biomarker in CLL. For this purpose, blood samples were collected from 90 CLL patients and 43 non-leukemic blood donors. Peripheral blood mononuclear cells (PBMCs) were isolated, total RNA was extracted and *in-vitro* polyadenylated, and first-strand cDNA was synthesized using an oligo-dT–adaptor primer. A real-time quantitative PCR assay was developed and applied for the quantification of i-tRF-GlyCCC in our samples. The biostatistical analysis revealed that i-tRF-GlyCCC levels are significantly lower in PBMCs of CLL patients, compared to PBMCs of non-leukemic controls, and that i-tRF-GlyCCC could be considered as a screening biomarker. Kaplan-Meier overall survival (OS) analysis revealed reduced OS for CLL patients with positive i-tRF-GlyCCC expression ($P = 0.001$). Multivariate Cox regression confirmed its independent unfavorable prognostic power with regard to OS. In conclusion, i-tRF-GlyCCC may constitute a promising molecular biomarker in CLL, for screening and prognostic purposes.

1. Introduction

Chronic lymphocytic leukemia (CLL) is a hematological malignancy and one of the most common types of leukemia in adults. It is characterized by the accumulation of abnormal B lymphocytes ($CD5^+$, $CD19^+$, $CD20^+$, $CD23^+$) in the blood, bone marrow, lymph nodes, and the spleen. Strong evidence of the occurrence of CLL represent chromosomal abnormalities of DNA such as deletion 13q, deletion 11q, and trisomy 12. The unmutated immunoglobulin heavy chain variable (*IGHV*) region, a high amount of leukemic B cells expressing ZAP-70 (20% or more) or CD38 (30% or more), high levels of serum beta-2-microglobulin, increased fraction of prolymphocytes in the blood, and rapid doubling time of lymphocytes are, also, considered as important prognostic factors [1,2].

Currently, there are two prognostic systems, which are, generally, used for the prognosis of CLL patients, namely the Rai and the Binet staging systems [3,4]. Additional factors which have a significant contribution to the formation of the CLL profile include patient's age, gender, ethnicity, lifestyle, and the exposure to certain chemicals [5,6]. Numerous studies have identified a high variety of prognostic biomarkers in CLL, including microRNAs (miRNAs). Our research group has previously identified miR-20b-5p [7], miR-92a-3p [8], and miR-155-5p [9] as molecular biomarkers in CLL.

tRNA-derived RNA fragments are small non-coding RNAs

Abbreviations: AUC, area under the curve; BCa, bias-corrected and accelerated; CD, cluster of differentiation; CI, confidence interval; CLL, chronic lymphocytic leukemia; CLL-IPI, chronic lymphocytic leukemia international prognostic index; HIF1A, hypoxia-inducible factor 1 alpha subunit; *IGHV*, immunoglobulin heavy chain variable region; i-tRFs, internal tRNA fragments; miR-155-5p, microRNA-155-5p; miR-20b-5p, microRNA-20b-5p; miR-92a-3p, microRNA-92a-3p; miRNA, microRNA; mRNA, messenger RNA; OS, overall survival; PBMC, peripheral blood mononuclear cell; qPCR, quantitative real-time PCR; ROC, receiver operating characteristic; RQU, relative quantification unit; *SNORD43*, small nucleolar RNA C/D box 43; *SNORD48*, small nucleolar RNA C/D box 48; tRNA, transfer RNA; tiRNAs, stress induced tRNA derived RNA fragments; tRFs, tRNA fragments; tsRNAs, toxic small tRNA derived RNA fragments; UTR, untranslated region; ZAP70, zeta-chain–associated protein kinase 70 kDa

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specifically cleaved from transfer RNAs (tRNAs), with a length of 14–48 nucleotides. They are classified into three main classes, namely the tRNA fragments (tRFs), the stress induced tRNA derived RNA fragments (tiRNAs), and the toxic small tRNA derived RNA fragments (tsRNAs), depending on the length and the origin [10]. The tRFs, with a length of 14–30 nucleotides, are further categorized in three series, namely tRF-5, tRF-3 (or 3′CCA tRF), and i-tRF [11]. tRF-5 and tRF-3 series derive from tRNA 5′ and 3′ ends of the mature tRNA transcript, respectively, and are cleaved in the cytoplasm [12,13]. i-tRFs constitute internal fragments of the mature tRNA transcripts [11]. The tsRNAs, also known as toxic small RNA fragments, are 16–48 nucleotides long, similar to tRF-5 and tRF-3, while the tiRNAs are 30–40 nucleotides long and are produced by a cleavage in the anticodon loop of the mature tRNA molecule [14].

The tRNA molecules present a high differentiation in their cleavage sites, thus leading to several tRNA derived RNA fragments varying in length and having distinct functions. They participate in translation regulation and gene silencing with a subsequent effect on cell viability and proliferation. Furthermore, they play a pivotal role in human diseases. The levels of some tRNA-derived RNA fragments differ between cancer and normal cells. In many types of cancer, including breast cancer [15], lung cancer [16], and prostate cancer [17], tRFs appear as significant regulators in the cancer profile and development, such as tumor aggressiveness and cancer cell metastasis. It has also been reported that particular tRFs are involved in the regulation of key cellular processes, such as DNA replication and repair, in a way similar to the regulation mediated by miRNAs [18]. Moreover, an important role of several tsRNAs has been demonstrated in CLL [19,20]. According to all these aforementioned findings, we assume that particular tRFs could be promising molecular biomarkers in various malignancies, including CLL.

Prompted by these indications, we investigated the prognostic and screening potential of a small number of tRNA-derived RNA fragments including i-tRF-GlyCCC, a fragment originating from tRNAs bearing the glycine anticodon CCC, in CLL. For the first time, a real-time quantitative PCR (qPCR) assay was developed, based on the SYBR® Green chemistry, for the relative quantification of i-tRF-GlyCCC against two housekeeping genes producing small nucleolar RNAs [21,22]. Our study included a cohort of 90 CLL patients with complete follow-up data and a control cohort of 43 non-leukemic individuals.

2. Materials and methods

2.1. Description of the study population

The study population consisted of 90 CLL patients and 43 non-leukemic blood donors, from the Hematology Unit of the Second Department of Internal Medicine in University General Hospital “Attikon” (Athens, Greece). The cohort of normal individuals was used as a control. Age and gender between the two cohorts did not differ significantly. 22 out of 90 CLL patients were subjected to treatment with either cyclophosphamide, vincristine sulfate and prednisone (16 cases) or chlorambucil and dexamethasone (6 cases). All patients had not received any treatment during at least six months before blood sample collection. The median time from initial diagnosis of CLL to blood sampling for the present study was 14 months (interquartile range = 2–33 months). After the collection, 34 patients received treatment, including fludarabine, cyclophosphamide, and rituximab. Binet and Rai staging systems are used to classify CLL patients in low-, intermediate- and high-risk groups. Patients with Rai stage 0 are considered as a low-risk group, while patients of stage I and II as an intermediate-risk group; stage III and IV patients are characterized as high-risk group.

All participants of the study signed a written informed consent. This study was approved by the Ethics Committee of the University General Hospital “Attikon” (Athens, Greece) and conducted according to the ethical standards of the Helsinki Declaration of 1975, as revised in 1983. Follow up information was collected, including dates and causes of death. The overall survival (OS) time was considered as the time from blood sampling to the date of death or last contact. Each death was considered as an event. It should be also noted that CLL-related causes (*e.g.* treatment toxicity, infections during overt CLL) accounted for all deaths recorded during the accrual follow-up period of the patients having provided sample for the current study. The median follow-up time was 54 months.

2.2. Cell collection and immunophenotyping

Peripheral blood mononuclear cells (PBMCs) were collected after blood centrifugation at a Ficoll-Hypaque gradient. Cell immunophenotyping was performed with a panel of evaluated monoclonal antibodies, as previously described [23]. The vast majority of collected patients’ PBMCs (> 90%) were leukemic B cells. According to an established threshold, cases with more than 30% of CD38-expressing cells were characterized as positive for this marker.

2.3. Cell line culture

The human chronic myeloid leukemia K-562 cell line was propagated appropriately in Iscove's Modified Dulbecco's Medium (IMDM), including 4 mM *L*-glutamine, 4500 mg/L glucose, and 1500 mg/L sodium bicarbonate. Cultures were maintained between 10^5 to 10^6 cells/mL and were incubated for two days in humidity at 37 °C, in 5% CO_2, until further use.

2.4. Nucleic acid extraction, RNA polyadenylation, and reverse transcription of poly(A) RNA

DNA and total RNA were extracted from isolated PBMCs and K-562 cells using the TRI Reagent® (Molecular Research Center, Inc. Cincinnati, OH). Purity and concentration of total RNA were determined with a BioSpec-nano Micro-volume UV–vis Spectrophotometer (Shimadju, Kyoto, Japan) and then stored in deep freeze (− 80 °C) for future use.

Total RNA was *in-vitro* polyadenylated using *E. coli* poly(A) polymerase and first-strand cDNA was synthesized, using an oligo-dT–adapter primer and MMLV reverse transcriptase.

2.5. Investigation of the IGHV mutational status

For the examination of the mutational status of the *IGHV* region, PCR amplification and Sanger sequencing were performed, as previously described [23]. *IGHV* genomic sequences were considered mutated when the homology with the closest germ line counterpart was less than 98%.

2.6. tRF analysis and primer design

The tRNA source of each tRF was determined using the MINTbase algorithm [24], by the alignment of sequencing reads, derived from sequencing data. Primers for specific tRFs were designed for those sequences that showed elevated representation in the aforementioned sequencing data.

2.7. Relative quantification of i-tRF-GlyCCC levels, based on real-time qPCR

A real-time qPCR assay, based on the SYBR® Green chemistry, applied in a 7500 Fast Real Time PCR System (Applied Biosystems, Foster City, CA, USA) was developed. The aforementioned assay was applied in all samples for the quantification of particular tRFs. Specific forward primers for the amplification of i-tRF-GlyCCC and the small nucleolar

RNAs *SNORD43* and *SNORD48* were designed and used along with a universal reverse primer, complementary with the oligo-dT–adapter, in the qPCR reactions.

The relative i-tRF-GlyCCC levels were determined using the comparative C_T ($2^{-\Delta\Delta Ct}$) method [25,26], and its prerequisites were checked in a validation experiment. The two aforementioned small nucleolar RNAs served as references and the cDNA from the K-562 cell line was the calibrator, thus rendering slight corrections – needed due to variation in the qPCR efficiencies among distinct runs – feasible [26]. The normalized i-tRF-GlyCCC levels of each sample were calculated as the ratio of i-tRF-GlyCCC molecules to the geometric mean of *SNORD43* and *SNORD48* molecules, divided by the same ratio previously calculated for the calibrator; at a last step, all normalized values were expressed as relative quantification units (RQU).

2.8. Biostatistical analysis

We performed extensive biostatistical analysis, including non-parametric Mann-Whitney *U* test, ROC curve, binary logistic regression, and survival analysis, including Kaplan-Meier analysis and Cox regression. For the determination of the optimal cut-off point and further categorization of patients into i-tRF-GlyCCC–positive and –negative ones, we used the X-tile software [27], an algorithm that enables the determination of the best cut-off value by correcting, for the use of minimum *P*-value statistics. This particular cut-off value was 0.036 RQU, equal to the 43rd percentile. For all statistical tests, the level of statistical significance was set at a probability value smaller than 0.050 ($P < 0.050$).

3. Results

3.1. Development and validation of a qPCR-based quantitative assay for i-tRF-GlyCCC

A real-time qPCR assay was developed for the relative quantification of i-tRF-GlyCCC against the mean *SNORD43* and *SNORD48* expression (Fig. 1A). The specificity of each amplification product was checked using melt curve analysis (Fig. 1B). Next, standard curves were constructed using serial dilutions of the K-562 cDNA, in order to validate amplification efficiencies; each reaction was performed in triplicates. The mean -ΔC_T values were plotted against log of cDNA dilutions (Fig. 1C).

The relative levels of i-tRF-GlyCCC were determined using the comparative C_T ($2^{-\Delta\Delta Ct}$) method. K-562 cDNA served as calibrator, while *SNORD43* and *SNORD48* were exploited as reference genes [22]. In order to normalize the i-tRF-GlyCCC levels in each sample, the ratio of i-tRF-GlyCCC molecules to the geometric mean of *SNORD43* and *SNORD48* molecules was calculated and then divided by the respective ratio previously calculated for the calibrator. At a last step, the normalized values were expressed as relative quantification units (RQU).

3.2. Description of the CLL patients' clinical features

The cohort of CLL patients included 64 males and 26 females, with a median age of 70.5 years (range: 50–90 years) at the time of diagnosis and sample collection. According to the Binet staging system, 53 patients (58.9%) were classified as stage A, 16 (17.8%) as stage B, and 21 (23.3%) as stage C. CD38 expression status was assessed to be positive for 16 (17.8%) cases, whereas the rest 74 (82.2%) patients were CD38-negative. The *IGHV* genomic locus was mutated in 46 (51.1%) samples, while 44 (48.9%) patients had unmutated *IGHV* sequences. The clinical features of the patient cohort are summarized in Table 1.

3.3. i-tRF-GlyCCC levels in PBMCs as a surrogate screening biomarker for CLL

The normalized i-tRF-GlyCCC levels ranged from 0.009 to 1.17 RQU with a mean ± SE of 0.078 ± 0.014 in the PBMCs (mostly leukemic B cells) of CLL patients, whereas the levels of this i-tRF were significantly higher in normal PBMCs ($P < 0.001$), as showed in Fig. 2A, varying between 0.022 to 1.26 RQU with a mean ± SE of 0.20 ± 0.036. The i-tRF-GlyCCC levels did not differ between male and female individuals. No correlation was observed between i-tRF-GlyCCC levels and patient age, white blood cell or lymphocyte count. Additionally, no association was observed between the i-tRF-GlyCCC expression status in CLL patients' PBMCs and CLL staging, serum LDH expression, CD38 expression in PBMCs, or the *IGHV* mutational status.

In order to elucidate the potential of i-tRF-GlyCCC as a surrogate screening biomarker, able to distinguish CLL patients from the non-leukemic population, we performed ROC and logistic regression analysis. As clearly depicted by the ROC curve in Fig. 2B, i-tRF-GlyCCC levels can discern CLL patients with remarkable specificity and sensitivity at the same time [area under the curve (AUC) = 0.80, 95% CI = 0.72 – 0.87, $P < 0.001$]. Additionally, univariate logistic regression analysis revealed that low i-tRF-GlyCCC levels could predict CLL occurrence (crude odds ratio = 32.1, 95% CI = 4.2–243.7, $P < 0.001$).

3.4. High i-tRF-GlyCCC levels as an independent, adverse prognostic factor in CLL

Long follow-up information and survival data were available for all patients participating in this study. 29 (32.2%) patients succumbed during the accrual follow-up period due to CLL-related causes. Based on the best prognostic cut-off value that was determined for i-tRF-GlyCCC using the X-tile algorithm, 39 CLL cases were classified as i-tRF-GlyCCC–negative and 51 as i-tRF-GlyCCC–positive. Nevertheless, it should be noted that several other cut-off points could be used for prognostic purposes as well, since the increased relative risk of CLL patients with higher i-tRF-GlyCCC levels is significant between the 25th and the 75th percentile of the distribution of the i-tRF-GlyCCC levels (data not shown).

Kaplan-Meier OS analysis revealed poor OS for i-tRF-GlyCCC–positive CLL patients ($P = 0.001$), compared to the i-tRF-GlyCCC–negative CLL patients (Fig. 3A). Moreover, univariate Cox regression analysis revealed a 4-fold increased risk of death for CLL i-tRF-GlyCCC–positive patients (HR = 3.95, 95%CI = 1.60–9.74, $P = 0.003$). Bootstrapping in Cox regression strengthened the conclusion that i-tRF-GlyCCC overexpression is a significant unfavorable prognosticator in CLL (HR = 3.95, 95%CI = 1.69–15.72, $P < 0.001$) (Table 2). Interestingly, the i-tRF-GlyCCC expression status could be used in a multiparametric prognostic model, along with CLL staging and CD38 expression status, mutational status, due to their independent prognostic value in CLL, which was revealed by multivariate Cox regression models (Table 3).

The CLL patients were further stratified into subgroups with distinct prognosis, according to the established prognostic factors also showing statistical significance in OS. Thus, stratified Kaplan-Meier OS analysis was performed for patients at Binet stage A, Rai stage I, intermediate risk, with unmutated *IGHV* sequence, and/or negative CD38 expression status. As presented in the respective Kaplan-Meier curves, i-tRF-GlyCCC–positive CLL patients at Binet stage A ($P = 0.004$) (Fig. 3B), Rai stage I ($P = 0.003$) (Fig. 3C), or intermediate risk ($P = 0.001$) (Fig. 3D), have lower OS probabilities than i-tRF-GlyCCC–negative patients with CLL at a similar disease stage and/or risk.

CLL patients with unmutated *IGHV* sequence and/or negative CD38 expression have a different prognosis from those with mutations in the *IGHV* locus or with higher numbers of CD38+ cells. In our study, high i-tRF-GlyCCC levels in patients with unmutated *IGHV* sequence were

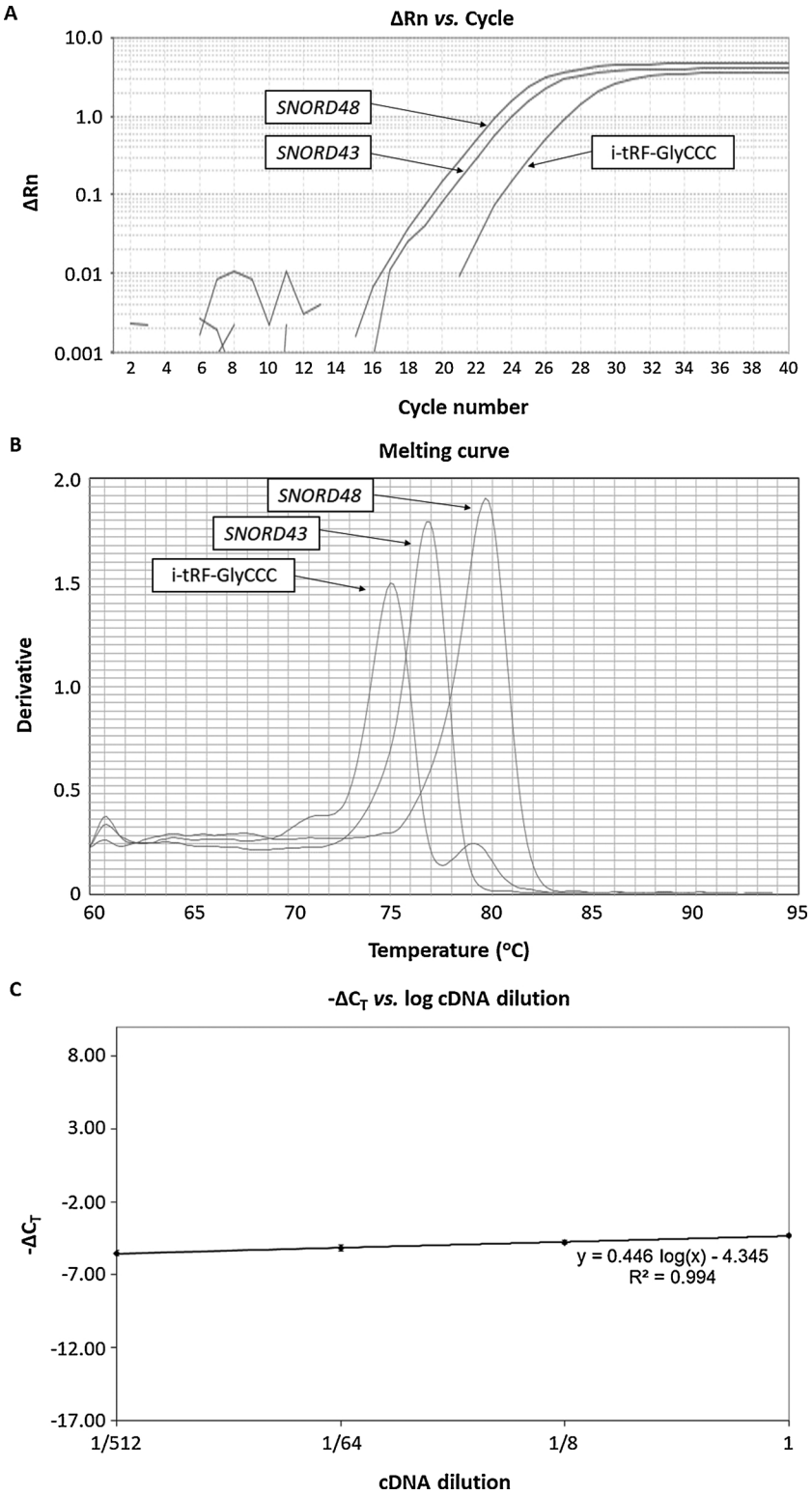

Fig. 1. Amplification plots, melt curves and a plot of -ΔC_T against log cDNA dilution, describing the i-tRF-GlyCCC, SNORD43 and SNORD48 amplification. **(A)** Amplification plots showing the amplification patterns of i-tRF-GlyCCC, *SNORD43* and *SNORD48*. **(B)** Melt curves illustrating the specificity of the amplified products of i-tRF-GlyCCC, *SNORD43* and *SNORD48*. **(C)** A curve illustrating the -ΔC_T against log cDNA dilution, in order to assess the efficiency of the amplification of i-tRF-GlyCCC, *SNORD43*, and *SNORD48*, and validate the comparative C_T ($2^{-\Delta\Delta CT}$) method.

related to an even more unfavorable prognostic outcome ($P = 0.004$) (Fig. 3E). On the other hand, patients exhibiting both a negative ($\leq 30\%$) CD38 expression and low i-tRF-GlyCCC levels seemed to have a prolonged OS, compared to the rest of patients with lower numbers of CD38+ cells ($P = 0.015$) (Fig. 3F).

4. Discussion

tRNAs are considered as molecules with leading roles in many cellular processes. In breast cancer, the expression of the initiator tRNA bearing the methionine amino acid is upregulated. This upregulation

Table 1
Clinicopathological characteristics of the CLL patients.

Total number of patients	90
Patient sex (Male/Female)	64/26
	Median (range)
Age (years)	70.5 (50–90)
OS (months)	40.5 (4–120)
White blood cells (x10^6/mL)	30,250 (2,000–96,900)
Lymphocytes (x10^6/mL)	21,965 (2,000–69,430)
CD38 expression (mean optical intensity of staining)	7.8 (0.1–59.2)
Binet stage	Number of patients (%)
A	53 (58.9%)
B	16 (17.8%)
C	21 (23.3%)
Rai stage	
0	17 (18.9%)
I	28 (31.1%)
II	24 (26.7%)
III	4 (4.4%)
IV	17 (18.9%)
Risk group	
Low	17 (18.9%)
Intermediate	52 (57.8%)
High	21 (23.3%)
Serum LDH concentration	
Normal	61 (67.8%)
Abnormal	29 (32.2%)
CD38 expression status	
Negative	74 (82.2%)
Positive	16 (17.8%)
***IGHV* mutational status**	
Mutated	46 (51.1%)
Unmutated	44 (48.9%)
Treatment[a]	
No	56 (62.2%)
Yes	34 (37.8%)

IGHV, immunoglobulin heavy chain variable; LDH, lactate dehydrogenase; OS, overall survival.
[a] Treatment after blood sample collection.

seems to affect the expression of other tRNAs and lead to increased proliferation and cell metabolism [28]. Their fragments have a critical role, as well. Even though the role of tRNA-derived RNA fragments in cellular processes and pathways remains enigmatic, great progress has very recently been achieved towards its clarification. These fragments appear as important regulators in numerous cellular processes, while several studies provide significant evidence supporting the implication of these molecules in cancer [29–31]. For instance, tRFs which are produced by Angiogenin may affect translation and promote the formation of stress granules in the majority of cancer types. Some tRFs have also been reported to contribute to the inhibition of apoptosome formation in cancer cells [12]. For instance, tRF/miR-1280 suppresses the proliferation and metastasis of stem-cell–like colorectal cancer cells [32], while in ovarian cancer tRF5-Glu decreases proliferation rates by inhibiting *BCAR3* expression, *via* binding to its 3′ untranslated region (UTR) [33].

In extracellular vesicles, high levels of tRNA-derived RNA fragments, combined with the already identified tumor-specific miRNAs, could be used for the distinction between tumor-derived vesicles and those with a different origin [34]. A relative example is reported in breast cancer cells, where some classes of tRFs are cleaved from tRNAs that carry the Glu, Asp, Gly, and Tyr amino acids. After their induction, these tRFs seem to suppress the stability of some oncogenic transcripts by displacing their 3′ untranslated regions (UTRs) from the RNA-binding protein YBX1 [15]. In CLL, some tRFs present differentiated levels. More precisely, the levels of ts-101 and ts-53 are downregulated in CLL; moreover, ts-53 binds to the 3′ UTR of *TCL1*, an oncogene with an important role in CLL, and regulates its expression in a miRNA-like way [35]. It has also been reported that these two tsRNAs may be mutated in CLL. Furthermore, a differentiation in the levels of particular tsRNAs has been observed not only between aggressive and indolent CLL, but also between indolent CLL patients and normal controls [19].

The existence of the i-tRF-GlyCCC, with the unique MINTbase code tRF-24-N3S3RQJV0X, was validated with qPCR and its sequence was submitted to GenBank with the accession number MK671732. This i-tRF is also expressed in uveal melanoma, skin cutaneous melanoma, breast invasive carcinoma, acute myeloid leukemia, colon adenocarcinoma, thyroid carcinoma, liver hepatocellular carcinoma, kidney renal clear cell carcinoma, and uterine corpus endometrial carcinoma.

In our study, we focused on the prognostic and discriminatory value of i-tRF-GlyCCC in CLL. In order to achieve this, an *in-house*–developed real-time qPCR assay was developed and applied in all our samples. The quantity of the target molecule was determined using the comparative C_T ($2^{-\Delta\Delta Ct}$) method. The calibrator cell line K-562 was used to avoid

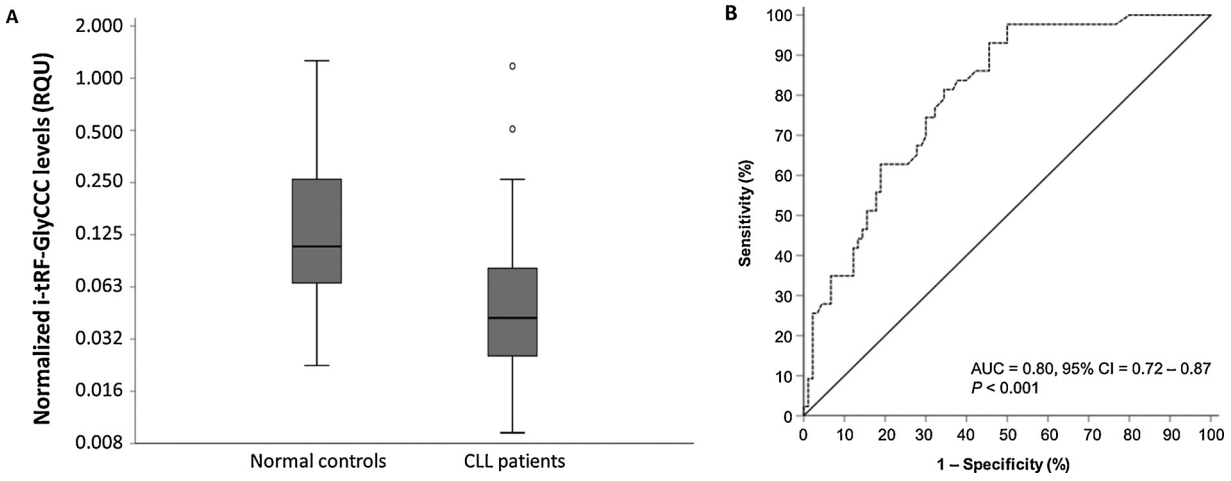

Fig. 2. i-tRF-GlyCCC levels in CLL patients and non-leukemic blood donors. (A) Box-plots show the lower levels of this molecule in CLL patients as compared to controls. **(B)** The ROC curve illustrates the potential of i-tRF-GlyCCC to distinguish CLL patients from non-leukemic population.

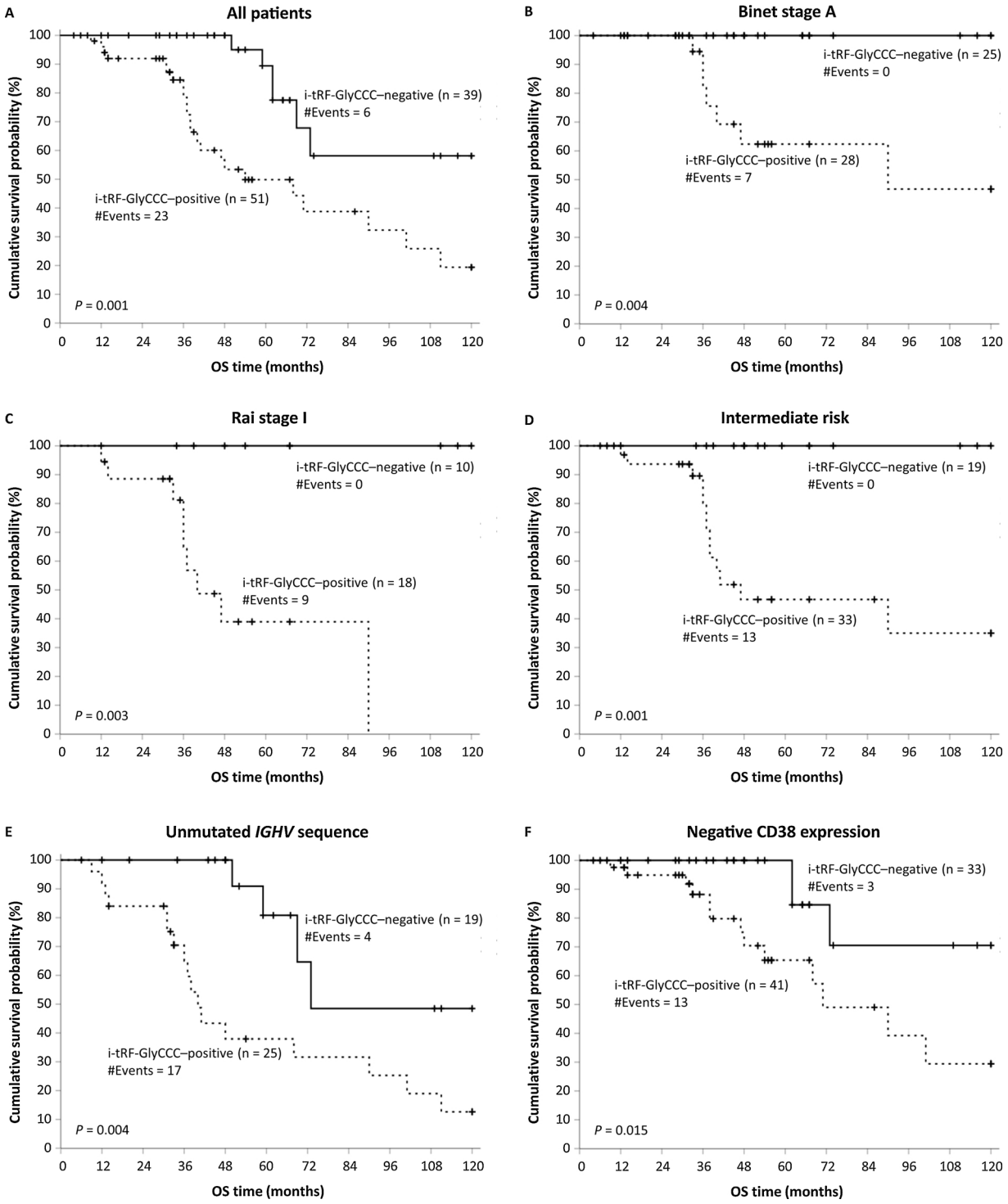

Fig. 3. Kaplan-Meier curves for the OS of CLL patients. Positive i-tRF-GlyCCC expression has an unfavorable prognostic value for **(A)** CLL patients, and particularly for those with **(B)** Binet stage A, **(C)** Rai stage I, **(D)** intermediate risk**, (E)** unmutated *IGHV* sequence, and/or **(F)** negative CD38 expression status.

differences in the amplification efficiencies of i-tRF-GlyCCC and the reference genes between distinct runs. The biostatistical analysis revealed the profound downregulation of i-tRF-GlyCCC in PBMCs (mostly leukemic B cells) of CLL patients, compared to PBMCs of non-leukemic controls. In fact, i-tRF-GlyCCC could be used as a surrogate screening biomarker, as revealed by the ROC curve.

From the Kaplan-Meier OS analysis, it became apparent that i-tRF-GlyCCC–positive patients had shorter OS time intervals compared to the i-tRF-GlyCCC–negative ones, and therefore, the unfavorable character of this i-tRF in CLL could be surmised. Its adverse prognostic impact is also confirmed by the univariate Cox regression and strengthened with bootstrap Cox regression analysis, as i-tRF-GlyCCC–positive patients are at a 4-fold higher risk for death due to disease-related causes, in comparison with patients with negative i-tRF-GlyCCC expression status. Besides i-tRF-GlyCCC levels, CLL staging and CD38 expression were strongly associated with OS time intervals; however, the prognostic value of i-tRF-GlyCCC is independent from the all other prognosticators, as shown by the multivariate Cox regression analysis. Based on these results as well as previous findings, we intend to build a multiparametric prognostic model composed of molecular biomarkers such

Table 2
Cox proportional hazard univariate regression analysis of i-tRF-GlyCCC expression and clinicopathological variables for the prediction of CLL patients' overall survival.

Covariate	HR	95% CI	*P* value[a]	BCa bootstrap 95% CI	Bootstrap *P* value[a]
i-tRF-GlyCCC expression (as continuous variable)	17.66	1.98– 157.5	**0.010**	0.14–275.4	**0.011**
i-tRF-GlyCCC expression					
Negative	1.00				
Positive	3.95	1.60–9.74	**0.003**	1.69–15.72	< **0.001**
Binet stage (ordinal)	2.02	1.34–3.03	< **0.001**	1.39–3.08	< **0.001**
Rai stage (ordinal)	1.46	1.11–1.91	**0.006**	1.16–1.91	**0.002**
Risk (ordinal)[b]	2.88	1.49–5.59	**0.002**	1.74–5.11	< **0.001**
CD38 expression					
Negative	1.00				
Positive	3.82	1.83–7.97	< **0.001**	1.74–12.38	< **0.001**
***IGHV* mutational status**					
Mutated	1.00				
Unmutated	2.20	0.97–4.98	0.060	0.99–5.87	**0.049**
Treatment					
No	1.00				
Yes	4.95	2.11–11.63	< **0.001**	2.18–19.55	< **0.001**

BCa, bias-corrected and accelerated; CI, confidence interval; HR, hazard ratio; *IGHV*, immunoglobulin heavy chain variable.
[a] The bold values indicate statistically significance of *P* values.
[b] Risk assessment is based on Rai staging and is used for determination of treatment options; CLL patients can be classified into low-, intermediate-, and high-risk groups.

as *KLKB1* mRNA [36], *HIF1A* mRNA [37], lipoprotein lipase mRNA [38], miR-20b-5p [7], miR-92a-3p [8] and miR-155-5p [9], and test its prognostic efficacy in a larger, independent cohort of CLL patients' samples.

The application of molecular biomarkers in clinical everyday practice is considered as a very important issue. Some potential points of interest would be the investigation of more CLL-IPI factors, such as the *TP53* mutational status and serum β_2-microglobulin concentration, together with the i-tRF-GlyCCC levels. Furthermore, the examination of i-tRF-GlyCCC importance in a higher number of patients from different ethnicities may result in highly significant information. It would also be tempting to investigate the efficiency of a multiparametric prognostic model in CLL, in which the i-tRF-GlyCCC along with other newly discovered molecular biomarkers could be integrated. Another point of research could focus on the investigation of a potential correlation between the type of treatment and i-tRF-GlyCCC expression, or even the possibility that this molecule could constitute a potential therapeutic target.

In conclusion, the aim of this study was to investigate the putative utility of i-tRF-GlyCCC in CLL as a molecular biomarker for prognostic and/or screening purposes. As a result, we identified the unfavorable prognostic value of i-tRF-GlyCCC in patients with CLL and its potential use in a multiparametric prognostic model, along with other established prognostic factors. Finally, i-tRF-GlyCCC could also be exploited as a surrogate screening biomarker, being able to distinguish with high sensitivity and specificity CLL patients from non-leukemic population.

Statement of ethics

This study was approved by the Ethics Committee of the University General Hospital "Attikon" (Athens, Greece) and conducted according to the ethical standards of the Helsinki Declaration of 1975, as revised in 1983. Informed consent was obtained from all individual participants included in the study.

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Authorship and disclosure

Contributions: KK performed experiments, collected and interpreted data, performed statistical analysis, and drafted the manuscript; PIA interpreted data and drafted the manuscript; SGP recruited patients, and collected clinicopathological and follow-up data; VP recruited patients; AS designed the research study and reviewed critically the manuscript; CKK designed the research study, performed experiments, analyzed data, performed statistical analysis, and reviewed critically

Table 3
Multivariate Cox regression analysis of i-tRF-GlyCCC expression and clinicopathological variables for the prediction of CLL patients' overall survival.

Covariate	HR	95% CI	*P* value[a]	BCa bootstrap 95% CI	Bootstrap *P* value[a]
i-tRF-GlyCCC expression (Positive *vs.* negative)	4.01	1.61–10.00	**0.003**	$1.49–4.9 \times 10^3$	**0.010**
Binet stage (ordinal)	1.66	1.07–2.59	**0.024**	0.93–5.26	0.052
CD38 expression (Positive *vs.* negative)	3.03	1.30–7.04	**0.010**	0.79–44.05	0.11
***IGHV* mutational status** (Unmutated *vs.* mutated)	1.19	0.46–3.05	0.72	0.43–4.12	0.71
i-tRF-GlyCCC expression (Positive *vs.* negative)	4.12	1.65–10.27	**0.002**	$1.43–2.4 \times 10^3$	**0.002**
Rai stage (ordinal)	1.28	0.94–1.73	0.12	0.82–2.67	0.23
CD38 expression (Positive *vs.* negative)	3.34	1.45–7.68	**0.005**	1–45.25	**0.048**
***IGHV* mutational status** (Unmutated *vs.* mutated)	1.18	0.46–3.06	0.73	0.43–3.44	0.73
i-tRF-GlyCCC expression (Positive *vs.* negative)	4.20	1.69–10.47	**0.002**	.	**0.009**
Risk (ordinal)[b]	2.22	1.07–4.57	**0.031**	0.85–14.16	0.077
CD38 expression (Positive *vs.* negative)	3.03	1.32–7.00	**0.009**	0.81–52.96	0.12
***IGHV* mutational status** (Unmutated *vs.* mutated)	1.21	0.48–3.08	0.69	0.41–3.95	0.68

BCa, bias-corrected and accelerated; CI, confidence interval; HR, hazard ratio; *IGHV*, immunoglobulin heavy chain variable.
[a] The bold values indicate statistically significance of *P* values.
[b] Risk assessment is based on Rai staging and is used for determination of treatment options; CLL patients can be classified into low-, intermediate-, and high-risk groups.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.leukres.2019.106234.

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