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Investigation of circulating lncRNAs in B-cell neoplasms

Mustafa Isin [a], Emre Ozgur [a], Guven Cetin [b], Nilgun Erten [c], Melih Aktan [d], Ugur Gezer [a], Nejat Dalay [a,*]

[a] Istanbul University, Oncology Institute, Department of Basic Oncology, Istanbul, Turkey
[b] Bezmialem Vakıf Gureba University, Faculty of Medicine, Department of Hematology, Istanbul, Turkey
[c] Istanbul University, Istanbul Faculty of Medicine, Department of Internal Medicine, Istanbul, Turkey
[d] Istanbul University, Istanbul Faculty of Medicine, Department of Hematology, Istanbul, Turkey

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ABSTRACT

Long non-coding RNAs (lncRNA) which are longer than 200 base pairs in length, play an important role in cellular machinery. Chronic lymphocytic leukemia (CLL) and multiple myeloma (MM) are neoplasms of B-cells. In our study we aimed to investigate circulating lncRNA levels of CLL and MM patients. For this purpose we selected 5 candidate lncRNAs (TUG1, LincRNA-p21, MALAT1, HOTAIR, and GAS5) where the first two are regulated by p53. Analyses were performed by real-time PCR using cDNA synthesized from plasma RNAs. In both disease groups differential levels of plasma lncRNAs were observed. LincRNA-p21 was the only molecule displaying significant changes in the CLL group while all remaining lncRNAs showed significant differences in the MM group. In the MM group only TUG1 showed higher levels than the healthy volunteers. In conclusion, the expression levels of the candidate lncRNA molecules display a general trend for tissue- and disease-specific expression which can provide important potential biomarkers specific to the particular disease type. However, further studies are necessary to elucidate their involvement in disease development and progression.

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1. Introduction

CLL being the disease of the elderly, is the most frequently observed leukemia in the western world, with a heterogeneous clinical outcome due to the genetic alterations in the leukemic cells. Leukemic cells display a wide diversity in their morphology, immunophenotype and genetic material. The chromosomal abnormalities most frequently observed in CLL are deletion in chromosome 13q, trisomy 12, and deletions in 11q, 17p, and 6q (4). In addition to these, expression levels of the zeta-chain associated (TCR) protein kinase 70 kDa (ZAP-70) or CD38 (adverse prognostic marker) and the presence of somatic *IGHV* mutations (patients carrying mutations respond better to the therapy) are widely investigated and are promising prognostic markers of the disease [1].

Multiple myeloma (MM) is another example of B-cell malignancies and is characterized by uncontrolled proliferation of plasma cell clones in the bone marrow. The disease may involve diffuse marrow infiltration, focal bone lesions and soft tissue (extramedullary) disease. MM is incurable and the main biomarker used for its diagnosis is monoclonal gammopathy in serum or urine samples of the patients. Staging of the disease is performed according to the International Staging System [2].

Gene expression data obtained from high throughput DNA microarrays have provided valuable information on the genes aberrantly expressed in MM. Furthermore, gene expression profiling and gene copy number alterations have shown a promising prognostic role that needs to be validated in larger studies. Besides, interactions between myeloma cells and bone marrow cells or extracellular matrix proteins that are mediated through cell surface receptors influence tumor growth, survival, migration, and drug resistance [2].

Protein-coding genes constitute only about 2% of the human genome. However, up to 70% of the human genome is transcribed into RNA [3]. Therefore, the human genome contains much more non-coding information than coding, in the form of a wide variety of non-coding RNA transcripts. In addition to classical “housekeeping” RNAs (e.g., ribosomal RNAs, transfer RNAs, and others) and the more recently discovered and well-defined microRNAs (miRNAs), the genome is also packed with long non-coding RNAs (lncRNAs). It has been shown that the mammalian genome encodes more than a thousand functional lncRNAs that have been mostly conserved across the mammals [3,4]. In a recent study 5446 lncRNA genes have been identified in the human genome and, combined with the previously published data this adds up to 6736 lncRNAs [5].

Due to their various structural and biochemical characteristics the lncRNAs are implicated in diverse biological functions, including nuclear architecture, regulation of gene expression, immune surveillance, or pluripotency of the embryonic stem cells. Recent studies provide

Abbreviations: CLL, Chronic Lymphocytic Leukemia; MM, Multiple Myeloma; LncRNA, Long non-coding RNA; TUG1, Taurine Up-regulated Gene 1; MALAT1, Metastasis Associated Lung Adenocarcinoma Transcript 1; HOTAIR, HOX Antisense Intergenic RNA; GAS5, Growth Arrest Specific 5; LincRNA-p21, Large intergenic non-coding RNA-p21.

* Corresponding author at: Oncology Institute, Istanbul University, 34093 Capa, Istanbul, Turkey. Fax: +90 212 5348078.
E-mail address: ndalay@yahoo.com (N. Dalay).

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evidence that lncRNAs are also involved in cancer development and maintenance of tumorigenesis [6,7]. However, the mechanism by which these lncRNAs function, and their regulation and role in cancer is unknown at present.

Contrary to the conventional belief that RNA is labile in nature, cell-free RNA is present and relatively stable in circulation [8]. It has been suggested that RNA in plasma or serum is associated with particulate matter such as apoptotic bodies or lipid vesicles and thus protected from degradation by nucleases [9,10]. Although a variety of RNA molecules including messenger RNA, and miRNAs have been investigated in the circulation so far, no data is available on the circulating lncRNAs and their relevance in disease.

The subject of the present study is cell-free lncRNAs in blood circulation and their significance in B-cell neoplasias. Since apoptosis and/or necrosis is a frequent event common to malignant tumors we hypothesized that lncRNA molecules are shed into circulation via vesicles and exosomes. To select the most appropriate candidate lncRNA molecules in B-cell neoplasia we analyzed the information available in the databases on the expression levels of the various lncRNA molecules in different cell and tissue types. As a result, five lncRNA molecules were selected according to their different functions in the tumor suppressor (LincRNA-p21 and GAS5), oncogenic (MALAT1 and TUG1) and epigenetic (HOTAIR) processes. HOTAIR is among the best-studied non-coding RNAs, and acts either as a *cis*- or *trans*-acting epigenetic regulator of chromatin [4]. It has recently been shown to promote invasiveness and metastasis in cancer [11,12] by reprogramming the chromatin state [13,11,14] and making it an important target for diagnosis and therapy. The metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is broadly expressed in human tissues [15] and known to be dysregulated in many human cancers. Expression of *MALAT1* correlates with tumor development, and progression or survival in lung, liver and breast cancer [16]. The taurine upregulated gene 1 (TUG1) has recently been shown to be transcriptionally regulated by p53 in response to DNA damage [13].

LincRNA-p21 and GAS5 exhibit tumor suppressor activities [17,18]. LincRNA-p21 has been named after its proximity to the p21 gene and acts as a transcriptional repressor. It forms a complex with the hnRNP-K ribonucleoprotein complex, and is involved in suppression of the cell cycle regulatory genes upon stimulation by the tumor suppressor protein p53 [17]. Expression of LincRNA-p21 is altered in certain conditions [19]. GAS5 (Growth Arrest Specific 5) has been first discovered in growth-arrested mouse NIH3T3 fibroblasts due to a significant increase in its expression level [18]. In humans it is transcribed from a small open reading frame on chromosome 1q25.1 which does not code for a functional protein [20,21]. Interaction of *GAS5* with the DNA binding domain of glucocorticoid receptors leads to the suppression of several antiapoptotic genes, rendering the cells sensitive to apoptosis [22]. Expression of GAS5 induces apoptosis in prostate and breast cancer cell lines. Lower GAS5 expression has been reported in breast tumors [23]. GAS5 is also a significant determinant in the mTOR pathway [24].

In this study, we investigated the circulating levels of the lncRNA molecules in plasma of the patients with chronic myeloid leukemia (CLL) and multiple myeloma (MM) and compared the levels with healthy subjects. This is the first report investigating the circulating long non-coding RNA levels in plasma of patients with chronic lymphocytic leukemia (CLL) and multiple myeloma (MM).

2. Materials and methods

2.1. Patients and healthy controls

68 patients with CLL, 62 patients with MM and 40 healthy individuals were enrolled in the study. The study was approved by the local ethics committee and written informed consent was obtained from the participants. Characteristics of the patients are given in Table 1.

Table 1
Characteristics of the patients.

CLL patients (n = 68)				MM patients (n = 62)	
RAI	Patient N (%)	BINET	Patient no. (%)	Durie–Salmon	Patient no. (%)
Stage 0	13 (18.8%)	A	19 (27.5%)	Stage 1A	4 (6.5%)
Stage I	7 (10.1%)	B	8 (11.6%)	Stage 2A	11 (17.7%)
Stage II	9 (13%)	C	5 (7.2%)	Stage 2B	4 (6.5%)
Stage III	2 (2.9%)	Unknown	37 (53.6%)	Stage 3A	17 (27.4%)
Stage IV	1 (1.4%)			Stage 3B	12 (19.4%)
Unknown	37 (53.6%)			Unknown	14 (22.6%)

Venous blood was drawn from patients and total RNA from 200 μl plasma was extracted using the TriPure Isolation Reagent (Roche, Germany).

RNA extraction from plasma was performed according to the protocol recommended by the manufacturer. Briefly, 1 ml TriPure Isolation Reagent was added to 200 μl plasma and homogenized. The homogenate was centrifuged at 12,000 *g* for 10 min at 4 °C. The supernatant was transferred into a clean tube, 0.2 ml chloroform was added and the tube was vortexed for 15 sec, and then incubated at RT for 10 min, followed by centrifugation at 12,000 *g* for 15 min. The colorless upper aqueous phase was transferred into a fresh tube and used for RNA extraction. RNA was precipitated from the aqueous phase by adding 0.5 ml isopropanol. The mixture was incubated at RT for 10 min and centrifuged at 12,000 *g* for 10 min at 4 °C. The supernatant was discarded and the RNA pellet was washed with 75% ethanol and centrifuged at 7500 *g* for 5 min at 4 °C. Following the air-drying, RNA was dissolved in RNase-free water, incubated at 55 °C for 15 min and stored at −80 °C until use.

The candidate lncRNAs were selected by searching the long non-coding database (www.lncrabd.org) and the Noncode v4 database. These databases report that HOTAIR, MALAT1, TUG1 and GAS5 are expressed in lymph nodes and white blood cells with the exception of LincRNA-p21 on which there is no data in the databases in human samples.

2.2. Quantification of lncRNAs

Total RNA isolated from plasma samples were used for cDNA synthesis using the First Strand cDNA Synthesis kit (Thermo Scientific, USA) according to the manufacturers' instructions. The real-time amplification of lncRNA molecules was performed using the LightCyler 480 (Roche, Germany). SYBR Green (Roche) was used as the fluorescent molecule. Primers sequences are shown in Table 2.

The endogenous reference gene for real-time PCR reactions was selected by comparing the basal expression levels of the GAPDH

Table 2
The primer sequences used in the study.

Gene	Primer sequence	Reference
HOTAIR	F 5′-GGCGGATGCAAGTTAATAAAAC-3′ R 5′-TACGCCTGAGTGTTCACGAG-3′	[13]
MALAT1	F 5′-GGATCCTAGACCAGCATGCC-3′ R 5′-AAAGGTTACCATAAGTAAGTTCCAGAAAA-3′	[25]
TUG1	F 5′-TAGCAGTTCCCCAATCCTTG-3′ R 5′-CACAAATTCCCATCATTCCC-3′.	[13]
LincRNA-p21	F 5′-GGGTGGCTCACTCTTCTGGC-3′ R 5′-TGGCCTTGCCCGGGCTTGTC-3′.	[17]
GAS5	F 5′-CTTCTGGGCTCAAGTGATCCT-3′ R 5′-TTGTGCCATGAGACTCCATCAG-3′.	[13]
GAPDH	F 5′-GCTCTCTGCTCCTCCTGTTC-3′ R 5′-ACGACCAAATCCGTTGACTC-3′	[13]

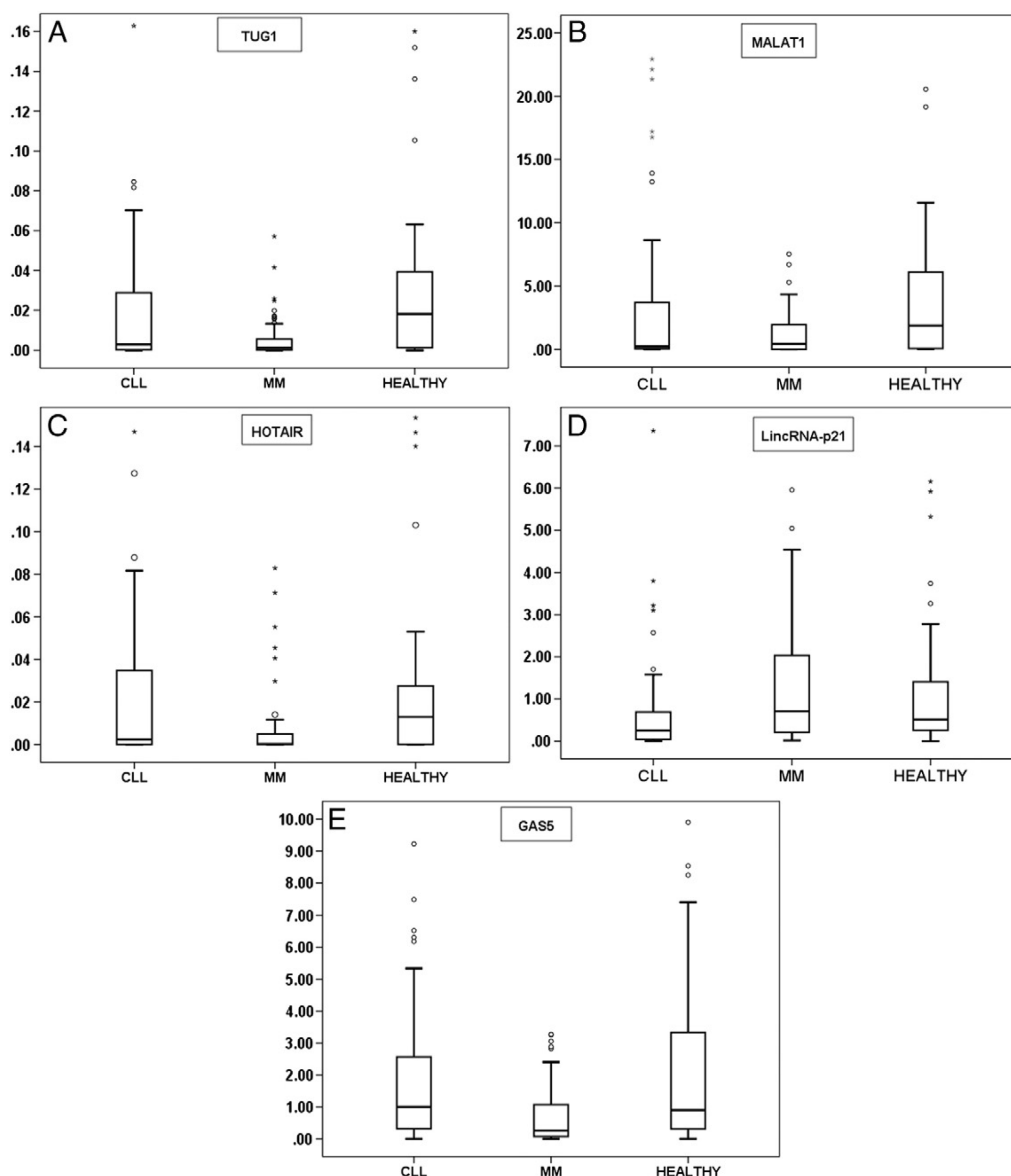

Fig. 1. Distribution of the lncRNA expression levels in the disease groups. A) TUG1, B) MALAT1, C) HOTAIR, D) LincRNA-p21, E) GAS5.

(glyceraldehyde-3-phosphate) and TBP (TATA box Binding Protein) genes. The GAPDH gene was chosen in view of its higher stability and consistency in the control plasma samples.

The PCR program included an initial "hot start" for 10 min, followed by 45 cycles of amplification. Each cycle consisted of a denaturation step at 95 °C for 10 s, annealing starting at 60 °C for 20 s and decreasing 2 °C every 2 cycles until 55 °C, and amplification at 72 °C for 30 s. For quantification of lncRNAs, the ΔΔCt method was used by coamplifying GAPDH for each sample and by comparing the Ct values. All experiments were performed twice and the mean values were calculated.

2.3. Statistics

Statistical analysis was performed using the SPSS v15 statistical package program.

3. Results

Plasma lncRNA levels were analyzed in 68 patients with CLL, 62 patients with MM and 36 healthy controls. We observed a significant difference in the expression levels of TUG1, MALAT1, HOTAIR and GAS5 lncRNAs between the patients with MM and the healthy controls. Interestingly, only LincRNA-p21 was differentially expressed in the CLL group (Fig. 1, Tables 3a and 3b). Cell-free lncRNA expression levels in the patients were lower than the healthy controls except for TUG1 expression which was upregulated in the plasma of MM patients. In addition, a strong association between the clinical stages and lncRNA expression levels were observed suggesting a correlation between plasma lncRNA levels and clinical subgroups (Table 3c). However, we failed to observe a correlation between GAS5 expression and the clinical stages in CLL patients (Table 3c). We also evaluated the sensitivity and specificity of these molecules as candidate biomarkers. Our data

Table 3a
Median values of the plasma lncRNA levels.

Median	CLL	MM	Healthy
TUG1	0.00296	0.12500	0.01808
MALAT1	0.24800	0.45100	1.89650
HOTAIR	0.00236	0.00022	0.01285
LincRNA-p21	0.25600	0.70700	0.51590
GAS5	0.99600	0.25300	0.89705

Table 3c
Correlation of plasma lncRNA levels with clinical stage.

Stage (p-value)	CLL	MM
TUG1	<0.001	<0.001
MALAT1	<0.001	<0.001
HOTAIR	<0.001	<0.001
LincRNA-p21	<0.001	<0.001
GAS5	0.10	<0.001

indicate that lncRNA molecules display significant differences in expression and that the majority of these act as inversely associated with the disease (Table 4).

4. Discussion

lncRNAs are implicated in numerous biological functions such as cell cycle control, apoptosis, imprinting and epigenetic regulation. They act in the modulation of transcription and play regulatory roles analogous to the proteins in controlling the localization and activity of proteins. LncRNAs function as scaffolds, microRNA sponges, competitors or activators for transcription, and take part in paraspeckles, splicing and endo si-RNA formation [26].

In this study we investigated expression levels of 5 candidate lncRNAs in plasma of patients with B-cell malignancies. To the best of our knowledge, this is the first study investigating free lncRNA levels in blood circulation of B-cell malignancies. In both patient groups we observed a distinct correlation between the cell-free lncRNA expression levels and the type of malignancy which indicates that lncRNA expression is unique to the cell type and reflects the lncRNA levels shed into circulation by the apoptotic or necrotic cells or microvesicular particles like exosomes [27]. This finding is in concordance with the study by Derrien et al. [28].

Two of our candidate molecules (LincRNA-p21 and GAS5) were tumor-inhibitory lncRNAs. LincRNA-p21 is involved in cell cycle regulatory pathways and its expression is regulated by the p53 gene. LincRNA-p21 expression was low in the patients with CLL. The low abundance of LincRNA-p21 may be explained by the lack of functional p53 protein. It is well known that deletions of chromosome 17p13 which harbors the p53 gene are a frequent event in CLL. In our patient group decreased plasma LincRNA-p21 levels correlated with the disease stage. This is also consistent with the fact that 17p13 deletions are more frequent during the late stages of CLL [29]. Expression of the other cell cycle arrest and apoptosis-related lncRNA, GAS5 was significantly lower in the patients with MM and slightly higher in the CLL group but in the latter case the difference was not significant. Downregulation of GAS5 is expected to inhibit apoptosis and allow the cells to maintain a more rapid cell cycle. Similar findings on the downregulation of the GAS5 gene have been reported for breast [23] or prostate [30] tumors and renal cell cancer [31]. Our data is also in concordance with a recent study indicating that GAS5 protects leukemic cells from the antiproliferative effects of chemotherapeutic agents [32]. The slight increase in the patients with leukemia is also consistent with the identification of GAS5 as a key regulator of lymphoid cell survival [33].

Although there are only a limited number of publications on TUG1, the information presented in the literature shows that TUG1 binds to polycomb repressive complex 2 (PRC2) and thereby affects the expression of cell cycle regulatory genes [13]. Knocking it down, affects retinal development in mice [34]. Higher TUG1 expression levels which were correlated with disease state have been observed in bladder carcinoma samples [35,13]. In accordance with this data it has been shown that downregulation of TUG1 inhibits proliferation of osteosarcoma cells and promotes apoptosis [36]. In our study, TUG1 levels were investigated in cell free plasma samples and higher expression was only observed in the MM group although correlation with disease state was observed both in the CLL and MM groups. Our data indicate that TUG1 may play a role in disease progression mainly in multiple myeloma.

Increased expression of MALAT1 has been reported in several cancers like lung [25], breast, colon and hepatocarcinoma [37,6,25,38,16,39]. In the patients with CLL, MALAT1 expression was higher but the difference was not significant. This is in concordance with a report that overexpression of MALAT1 may induce the cells to enhanced proliferation and also in line with the reports suggesting that MALAT1 expression is tissue-specific [40,41]. Our data suggest that MALAT1 expression in B-cell malignancies is lower than it is in the solid tumors. We observed significantly lower MALAT1 expression levels in the circulation of MM patients. This may indicate an aberrant splicing machinery since MALAT1 is involved in splicing [42] and its differential expression has been linked with metastasis and recurrence [25,43].

Chromatin remodeling plays an important role in tumorigenesis since during this process cell cycle-regulatory genes are affected. HOTAIR plays an important role in chromatin remodeling by interacting with PRC2 and H3K27 and repressing the HOXD locus [44]. In contrast to the studies reporting high HOTAIR expression levels in malignancies like primary breast, lung, hepatocellular or colorectal carcinomas and gastrointestinal stromal tumors [11,45–47] in our study group the patients with MM displayed lower plasma HOTAIR levels. This supports the view that the effect of HOTAIR may be tissue-specific [48]. It has been shown that the association between the expression of several lncRNAs and cell proliferation are species and/or tissue-dependent [49] and that overexpression of HOTAIR does not facilitate proliferation of lung cancer cells [41].

Table 3b
Significance of plasma lncRNA levels in distinguishing the disease groups.

Patients vs. healthy (p-value)	CLL (n = 68)	MM (n = 62)
TUG1	0.106	0.0002
MALAT1	0.109	0.0012
HOTAIR	0.426	0.0016
LincRNA-p21	0.018	0.76
GAS5	0.922	0.0077

Table 4
AUC (area under curve) values of lncRNAs in the two disease groups.

Candidate LncRNA	Area under curve (AUC) (CI = %95)	
	CLL	MM
TUG1	0.404	0.274
MALAT1	0.411	0.303
HOTAIR	0.452	0.309
LincRNA-p21	0.360	0.519
GAS5	0.506	0.338

5. Conclusion

In conclusion, the expression levels of the candidate lncRNA molecules display a general trend for tissue- and disease specific expression which can provide important potential biomarkers specific to the particular disease type. However, further studies are necessary to elucidate their involvement in disease development and progression.

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