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Elevated serum lncRNA TUG1 levels are a potential diagnostic biomarker of multiple myeloma

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Long noncoding RNAs (lncRNAs) have increasingly been found to be key mediators of tumor biology and to have potential diagnostic value as biomarkers of particular forms of cancer. TUG1 (taurine-upregulated gene 1) is an lncRNA that has been found to be upregulated in a range of different cancer types, but levels of its expression in the serum of patients with multiple myeloma (MM) are uncertain, as is its diagnostic relevance in such a population. This study therefore explored whether TUG1 levels in patient serum serve as a diagnostic biomarker of MM. We analyzed serum TUG1 levels via quantitative real-time polymerase chain reaction in healthy control and MM patient serum and observed clear TUG1 upregulation in MM patients ($p < 0.001$). We further found that the levels of TUG1 in patient serum correlated with factors including disease clinical stage, β_2-microglobulin, total protein, albumin, globulin, and bone injury ($p < 0.05$), suggesting that this lncRNA may be independently predictive of MM disease stage. We found areas under receiver operating characteristic curves as high as 0.792 ($p < 0.001$) for TUG1—a value higher than that for either β_2-microglobulin (0.747) or albumin (0.597)—with the combination of all three biomarkers improving diagnostic specificity and areas under the curve of 96.9% and 0.836, respectively ($p < 0.001$). Together, our results suggest that serum TUG1 levels may serve as a valuable biomarker that can help to facilitate MM diagnosis. **© 2019 ISEH – Society for Hematology and Stem Cells. Published by Elsevier Inc. All rights reserved.**

Multiple myeloma (MM) is a form of cancer in which bone marrow plasma cells undergo malignant transformation [1,2]. In individuals with MM, these plasma cells proliferate abnormally and produce high levels of the monoclonal immunoglobulin light-chain (M)-protein, although a limited subset of patients have a nonsecretory form of MM in which this M-protein is not produced. Prior to the onset of MM, patients often have monoclonal gammopathy of undetermined significance (MGUS) [3] and smoldering multiple myeloma (SMM) [4]. The primary symptoms with which MM patients present are hypercalcemia, anemia, bone lesions, and renal failure [5]. Many different efforts in recent years have helped to develop a range of treatment strategies for MM patients, including immunomodulatory agents and inhibitors of the proteasome. These new compounds, together with high-dose chemotherapy, corticosteroids, other traditional treatments, and autologous hematopoietic stem cell transplantation, have allowed for an increasing number of patients to attain a long-term clinical response to therapy [6]. The SMM and MGUS states that precede MM, however, are typically not symptomatic and are most often detected only incidentally while patients are being assessed for an unrelated condition [5]. There is thus a

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need for the identification of novel biomarkers of these MMs, with genome-wide transcriptomic data sets being ideal for such biomarker identification.

Although the majority of the genome has been found to be transcribed, only 2% of it or less actually encodes for protein, suggesting that a large amount of RNA lacks coding potential [7]. A range of different noncoding RNA (ncRNA) species have been identified, including microRNAs (miRNAs), long ncRNAs (lncRNAs), and circular RNAs (circRNAs), with all of these species playing regulatory roles in a variety of physiological contexts [8]. The processes regulated by these molecules are in some cases unique to particular species, whereas in other cases they are highly evolutionarily conserved. LncRNAs are >200 nucleotides long [9], and have been reported to be capable of regulating differentiation [10], proliferation [11], apoptosis [12], and cancer progression [13]. These lncRNAs can also persist in the urine or serum of humans, and as such they have potential value as therapeutic or diagnostic biomarkers in patients being assessed for a given disease state in which they are known to be dysregulated [14–16]. MM patients have previously been found to exhibit elevated levels of particular lncRNAs such as PRINS [17], MALAT1 [18], and H19 [19], and these lncRNAs have been ascribed pro-oncogenic functions. Certain other lncRNAs, however, are downregulated in MM patients, including MEG3 [20] and NEAT1 [21], and these have been associated with cancer progression. Whether serum lncRNAs are a reliable and clinically relevant biomarker of MM, however, is uncertain at present.

TUG1 (taurine-upregulated gene 1) is an lncRNA that is 7.1 kb long that is most normally expressed in retinal and brain tissues, as it was originally detected in a screen for genes that were induced in response to taurine treatment of retinal cells. TUG1 is essential for normal retinal photoreceptor development [22], but it has also been reported to be dysregulated in the context of oncogenesis, suggesting it may play a role in cancer [23]. TUG1 expression was shown to be upregulated in both glioma stem cells (GSCs) [24] and hepatocellular carcinoma (HCC) cells [25], whereas its levels were reduced in non-small cell lung cancer (NSCLC) [26] and bladder cancer [27]. Altered TUG1 levels are linked to a range of disease-related processes including cell growth, apoptotic death, and metastasis [28]. Here, we explore the diagnostic and clinical relevance of serum TUG1 in MM patients to highlight its potential use as an auxiliary serum biomarker suitable for MM diagnosis.

Methods

Sample collection

Serum was collected from 110 patients with newly diagnosed MM, as well as from 98 healthy age-matched controls, at the Affiliated Hospital of Nantong University (Nantong, China) from March 2017 to June 2018. Sample size was calculated as follows: $n = (Z_{\alpha/2} + Z_{\beta})^2\sigma^2/\varepsilon^2$, where $\alpha = 0.05$, $1 - \beta = 0.80$, and the sample standard deviation s is used instead of σ (http://www.blueballon.cn/Sample/index.html). We additionally combined 50 normal samples that were from healthy controls for qRT-PCR experiments and confirmation of methodological quality. All patient samples were obtained on the second day after admission to the hospital. Following collection, blood was spun for 10 min at 1,000*g*, and standard clinical protocols were used to assess other biochemical parameters within patient serum samples. We also collected bone marrow samples from 26 patients with newly diagnosed MM and 22 healthy donors. Ficoll (GE Healthcare Bio-Science, USA) was used to isolate the $CD138^+$ mononuclear cells from these bone marrow samples, and was followed by the use of CD138 microbeads (Miltenyi Biotec, Germany). Serum and $CD138^+$ cell samples were stored at −80°C in tubes free of RNase prior to their use. The Human Research Ethics Committee of the Affiliated Hospital of Nantong University approved this study.

RNA extraction and cDNA synthesis

A total of 400 μL of patient serum was used with a serum extract kit (Bioteke, Beijing, China) to isolate total RNA, whereas Trizol was used to isolate RNA from $CD138^+$ cells based on provided protocols. A spectrophotometer (NanoPhotometer, IMPLEN, Germany) was used to determine RNA concentrations, after which 10 μL of RNA from each sample was used with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) to generate cDNA. Each reaction contained 10 μL of total RNA, 4 μL of 5 × reaction buffer, 2 μL of 10 mmol/L dNTPs, 1 μL of oligo(dT) primer, 1 μL of RNase inhibitor (20 U/μL), 1 μL of reverse transcriptase (200 U/μL), and 1 μL of RNase-free water. Reactions were heated to 42°C for 60 min and then to 70°C for 5 min. cDNA was stored at −80°C when not in use.

qRT-PCR

A LightCycler 480 (Roche, Switzerland) was employed for all reactions, using 3 μL of cDNA per reaction. Individual reactions contained a total of 10 μL of SYBR Green I Mix (Roche), 1 μL of each primer, and 5 μL of RNase-free H_2O. Thermocycler steps were as follows: 95°C for 5 min, then 45 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. All primers are compiled in Supplementary Table E1 (online only, available at www.exphem.org). The $2^{-\Delta\Delta CT}$ method was used to analyze relative TUG1 expression.

Cell culture

The H929, U266, and RPMI8226 MM cell lines were from the Chinese Academy of Sciences Cell Bank (Shanghai, China), and were grown using RPMI-1640 (Corning, USA) containing 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and penicillin/streptomycin (Gibco) at 37°C in a humidified 5% CO_2 incubator. Normal plasma cells were isolated from the bone marrow of healthy donors.

Bioinformatics analyses

MM microarray profiles were from the GEO database (http://www.ncbi.nlm.nih.gov/geo/), with the GSE5900 and GSE116294 data sets being used for analyses herein. TUG1 expression was analyzed with an R package. Differences in TUG1 levels between plasma cell leukemia (PCL) patients and controls were assessed via nonparametric *t* tests, with $p < 0.05$ considered to indicate statistical significance.

Detection of other relevant indicators

Serum β_2-microglobulin (β_2-MG) and albumin (ALB) were assessed using an ADVIA2400 (Siemens, USA), with the following cutoff values used to distinguish positive and negative results: 2.95 μg/mL for β_2-MG, 34.95 g/L for ALB. Cytogenetic assays were used to detect IgH translocation, p53 deletion, 13q14 deletion, RB1 deletion, and 1q21 amplification using fluorescence in situ hybridization (FISH).

Statistical analysis

SPSS version 22.0 (IBM, Armonk, NY) was used for all testing. Graphpad 8.0 was used for figure generation. Serum levels in MM patients and controls were compared via independent sample *t* tests, with one-way analyses of variance and χ^2 tests being used as appropriate. Logistic regression analyses were used for univariate and multivariate testing. Receiver operating characteristic (ROC) curves were analyzed to assess the area under the curve (AUC), specificity, and sensitivity of serum TUG1, β_2-MG, and ALB individually and in combination using multiple logistic regression analysis. The optimal diagnostic cutoff for this signature was assessed based on the cutoff value with the largest Youden's index (sensitivity + specificity − 1) [29]. The significance threshold was $p < 0.05$.

Results

TUG1 identification

We initially analyzed the serum samples from 20 healthy controls and 20 MM patients to evaluate TUG1 levels, which we found to be significantly increased in those with MM (Figure 1A). We further confirmed that TUG1 levels were similarly increased in both bone marrow samples (26 MM patients and 22 healthy controls) and MM cell lines (H929, U266, RPMI 8226) relative to appropriate controls (Figure 1B, C).

Serum TUG1 detection

As serum samples can be collected in a minimally invasive manner, they represent an ideal means of screening for diagnostic biomarkers. However, there is currently no standardized means of screening patients for serum lncRNA expression levels. We therefore assessed the levels of five possible reference genes in the serum of 5 control and 7 MM patients: 18 S ribosomal RNA (18 S rRNA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin (ACTB), polymerase (RNA) II (RPII), and α-tubulin (TUB) (Supplementary Table E2, online only, available at www.exphem.org). We found the 18 S rRNA to be the most highly expressed of these genes, with no significant difference between MM patients and healthy donors (Supplementary Figure E1, online only, available at www.exphem.

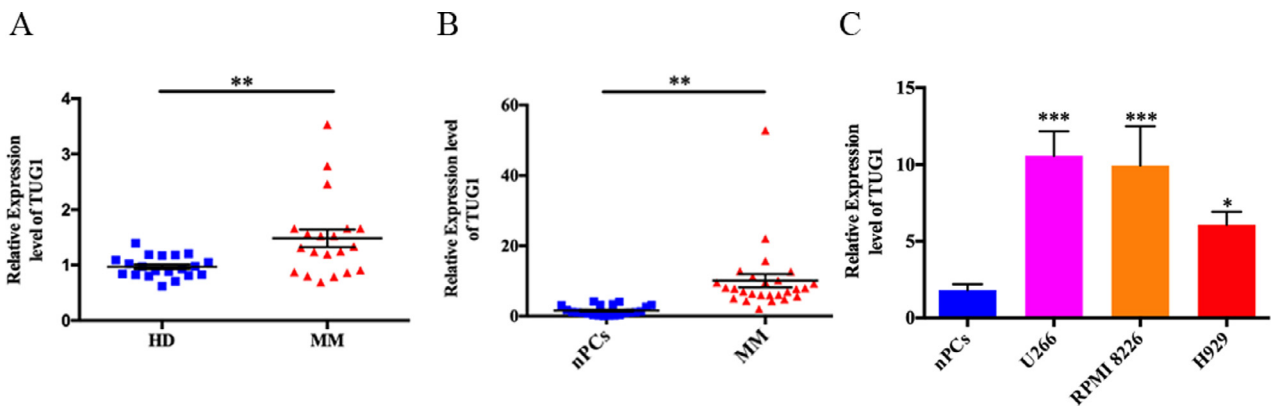

Figure 1. Long non-coding RNA TUG1 identification. **(A)** Serum TUG1 levels in MM patients ($n = 20$) and healthy controls ($n = 20$). **(B)** Levels of TUG1 in bone marrow samples from MM patients ($n = 26$) and healthy controls ($n = 22$). **(C)** MM cell line TUG1 levels. **(A, B)** Nonparametric *t* test. **(C)** One-way analysis of variance. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. nPCs: normal plasma cells isolated from non-patient controls.

org), and as such we used this gene as a reference. Afterward we assessed our ability to specifically detect TUG1 based on melting curve, PCR sequencing, and agarose gel electrophoretic analyses (Supplementary Figure E2A–C, online only, available at www.exphem.org). Through these approaches, we confirmed accurate TUG1 detection based on the presence of a single melting curve peak, a single electrophoretic band, and a correct sequence. We next assessed the degree to which we were able to linearly detect TUG1 levels via diluting TUG1 cDNA through a series of 10-fold dilutions, revealing an R^2 of 0.9969 for the resultant standard curve, confirming that qRT-PCR was well-suited to reliable serum TUG1 detection (Supplementary Figure E2D). We also assessed the stability of TUG1 in serum samples by leaving these samples at room temperature for 0–24 h or by freeze-thawing them 0–10 times prior to qRT-PCR analysis. We observed no appreciable decrease in levels of serum TUG1 in response to these changes (p>0.05) (Supplementary Figure E2E, F). We also obtained satisfactory intra- and inter-assay coefficients of variation (CVs) for TUG1 (Table 1).

MM patients exhibit increased serum TUG1 levels

We next used qRT-PCR as a means of confirming the relationship between TUG1 levels and MM through the analysis of serum samples from 98 healthy controls and 110 MM patients, revealing a significant increase in TUG1 levels in MM patient serum ($p < 0.001$; Figure 2A). Using data in the GEO database, we also assessed TUG1 levels in patients with MGUS, SMM, and PCL, revealing that there was no significant difference in MGUS or SMM patient TUG1 levels ($p > 0.05$, Figure 2B), although it was significantly elevated in those with PCL ($p < 0.05$, Figure 2C).

Table 1. Intra- and inter-assay reproducibility of TUG1 and 18 S RNA

	TUG1	18 S RNA
Intra-assay		
Mean ± SD	27.80 ± 0.23	21.03 ± 0.17
CV	0.82%	0.80%
Inter-assay		
Mean ± SD	27.09 ± 0.96	20.15 ± 0.88
CV	3.56%	4.35%

TUG1 is associated with MM clinical stage

We next stratified the 110 MM patients analyzed in this study on the basis of their staging according to the following systems: Durie–Salmon (DS), International Staging System (ISS), and Revised International Staging System (R-ISS). We then analyzed TUG1 serum levels in patients as a function of disease stage, revealing that DS-III ($n = 84$) patients had significantly elevated TUG1 levels as compared with DS-I ($n = 13$) and DS-II ($n = 13$) patients ($p < 0.05$), whereas there was no significant difference in levels between the latter two cohorts ($p > 0.05$) (Figure 3A). Similarly, ISS-III ($n = 37$) patients had higher serum TUG1 levels than did ISS-I ($n = 21$) or ISS-II ($n = 52$) patients ($p < 0.05$), with no significant difference between the latter two cohorts ($p > 0.05$) (Figure 3B). R-ISS-II ($n = 55$) patients also had significantly higher serum TUG1 than did R-ISS-I ($n = 15$) patients ($p < 0.05$), while levels in R-ISS-I and ISS-II ($n = 40$) patients, or between R-ISS-II and ISS-III patients, did not differ substantially ($p > 0.05$) (Figure 3C). This suggests that TUG1 levels in MM patient serum may be of value for staging disease.

Serum TUG1 correlated with MM clinicopathological findings

We next associated the relationship between serum TUG1 and clinicopathological findings in patients by separating these 110 MM patients on the basis of median serum TUG1 levels into TUG1-high ($n = 56$) and -low ($n = 54$) groups. The relationships between TUG1 levels and sex, age, M-protein, β_2-MG, renal

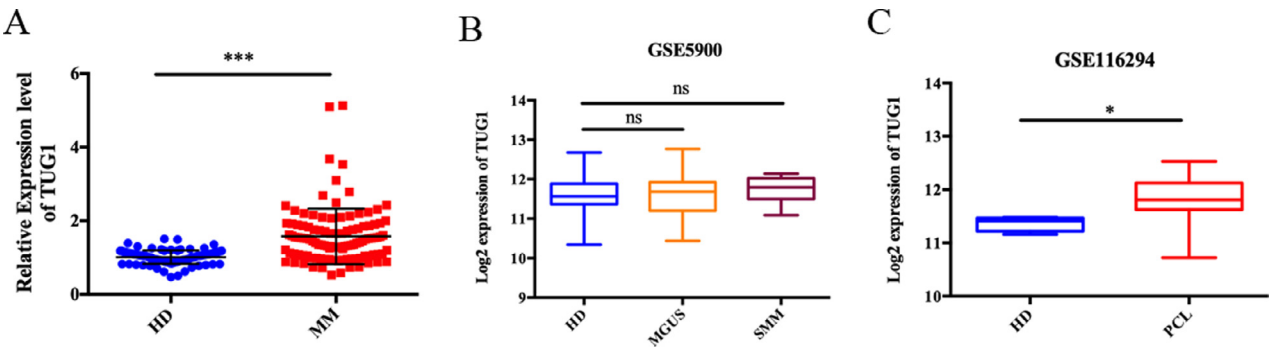

Figure 2. Serum TUG1 is increased in MM patients. **(A)** Levels of serum TUG1 in healthy controls ($n = 98$) and newly diagnosed MM patients ($n = 110$). **(B)** TUG1 levels in those with MGUS and SMM was determined based on GEO data sets. **(C)** TUG1 levels in PCL patients were determined based on GEO data sets. **(A)** Independent sample t test. **(B, C)** Nonparametric t test.

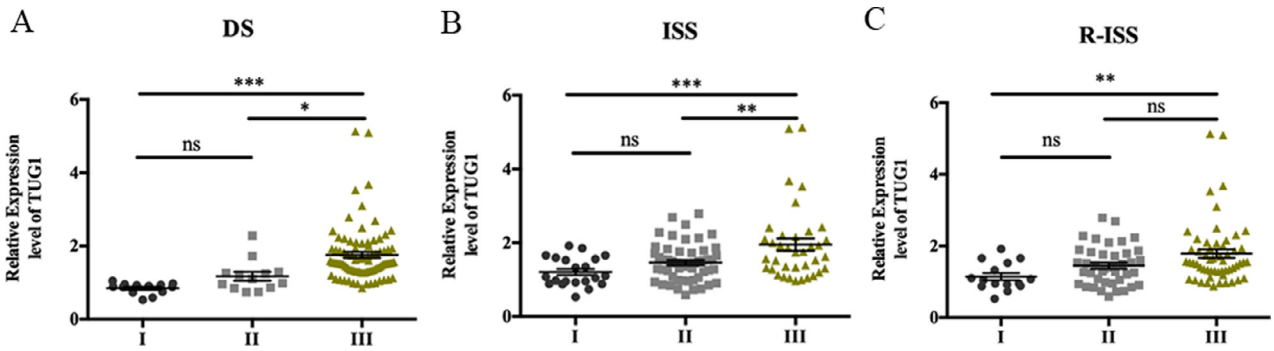

Figure 3. TUG1 expression is associated with MM clinical stage. TUG1 expression differed as a function of **(A)** DS stage, **(B)** ISS stage, and **(C)** R-ISS stage. **(A–C)** One-way analysis of variance. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

injury, bone injury, and other factors were then assessed. We found elevated serum TUG1 to be significantly correlated with total protein ($p = 0.035$), ALB ($p = 0.037$), globulin ($p = 0.035$), and β_2-MG ($p = 0.037$) (Table 2). There was also a significant association between bone injury and serum TUG1 ($p = 0.007$). Sex, age, and renal injury were not correlated with serum TUG1 levels ($p > 0.05$).

Analysis of factors independently associated with MM patient DS stage

We next conducted a univariate analysis to identify clinicopathological findings associated with MM DS stage, including serum TUG1, β_2-MG, M-protein, light chain, HGB, total protein, ALB, globulin, renal injury, and bone injury levels, with results given in Table 3. We found that β_2-MG ($p = 0.005$), ALB ($p = 0.038$), renal injury ($p = 0.019$), bone injury ($p < 0.001$), and TUG1 ($p < 0.001$) were all significantly associated with MM patient DS stage. We then incorporated these variables into a multivariate analysis, which revealed that β_2-MG ($p = 0.015$), bone injury ($p = 0.001$), and serum TUG1 ($p < 0.001$) were all independently associated with MM patient DS stage.

Assessment of TUG1, β_2-MG, and ALB diagnostic utility in MM patients

We next generated ROC curves to assess the potential diagnostic utility of serum TUG1 in MM patients and found that TUG1 levels were able to differentiate between MM patients and controls with an ROC AUC of 0.792 (95% confidence interval [CI]: 0.729–0.855; $p < 0.001$) (Fig. 4A). This was superior to the diagnostic value of the more traditional markers β_2-MG (Fig. 4B) and ALB (Fig. 4C). The combination of multiple markers has been reported to enhance diagnostic accuracy [30], and as such we explored whether combining TUG1, β_2-MG, and ALB might improve MM diagnosis. Individually, TUG1 had sensitivity and specificity values (65.5% and 94.9%) that were significantly higher than those for β_2-MG (65.5%, 79.6%) and ALB (54.5%, 69.4%) (Table 4), giving TUG1 an overall diagnostic accuracy of 79.3%, as opposed to 72.1% for β_2-MG and 58.6% for ALB. When these three markers were combined, diagnostic specificity and AUC were further improved to 96.9% and 0.836 (95% CI: 0.779–0.894, $p < 0.001$) (Table 4).

Discussion

MM remains the second most frequent hematological malignancy, with an age-adjusted incidence of 6 per 100,000 annually in the United States and Europe, and an as yet hard-to-treat disease, manifesting worldwide, particularly in older populations [31]. Traditionally, MM diagnosis depends on the detection of abnormal antibodies in serum or urine tests, imaging evidence of bone lesions, and bone marrow biopsy results [5]. In its early stages, MM lacks apparent symptoms, making it essential that an effective screening approach for detecting those with early disease be developed. Tumor biomarkers that are absent in normal tissue but present in those with certain cancers represent ideal screening markers, as they can offer insight into tumor type, allowing doctors to more readily determine patient diagnosis, prognosis, and treatment options [32].

Altered lncRNA expression has increasingly been associated with a range of cancer types including MM. For example, in colorectal cancer (CRC), Abedini et al. found that serum levels of the lncRNAs ATB and CCAT1 were elevated, and these serum lncRNAs could be reliably used to differentiate those with CRC from healthy controls [33]. In a similar vein, Tan et al. found increased serum HOTAIR levels in those with glioblastoma multiforme (GBM) relative to controls, with an AUC of 0.913 and markedly elevated HOTAIR levels in those with particularly high grade tumors [16]. In an analysis of MM patients, Taiana et al. found the lncRNA NEAT1 to be increased in MM patients, making it a potentially valuable diagnostic or therapeutic biomarker in this disease [34]. Together, these

Table 2. Correlation between TUG1 expression and clinical characteristics of MM patients

Clinical characteristic	Case no.	TUG1 expression		Pearson's χ^2	p value
		Low expression	High expression		
Sex				0.350	0.554
Male	60	31	29		
Female	50	23	27		
Age, y				1.295	0.255
<65	53	29	24		
≥65	57	25	32		
M-protein				0.009	0.926
IgA	31	15	16		
IgG	79	39	40		
Light chain				1.760	0.185
Λ	58	25	33		
K	52	29	23		
Hemoglobin				0.010	0.920
<120 g/L	86	42	44		
≥120 g/L	24	12	12		
Total protein				4.464	0.035[a]
Abnormal value	68	28	40		
Normal value	42	26	16		
Albumin				4.371	0.037[a]
Abnormal value	62	25	37		
Normal value	48	29	19		
Globulin				4.464	0.035[a]
Abnormal value	68	28	40		
Normal value	42	26	16		
β_2-Microglobulin				4.354	0.03[a]
<3.5 μg/mL	53	31	22		
≥3.5 μg/mL	57	22	35		
Renal injury[b]				0.598	0.439
Yes	83	39	44		
No	27	15	12		
Bone injury[c]				7.349	0.007[d]
Yes	69	27	42		
No	41	27	14		

Statistical analyses were carried out using Pearson's χ^2 test.
[a]$p<0.05$, statistically significant.
[b]Renal insufficiency: creatinine clearance <40 mL/min or serum creatinine >177 μmol/L (>2 mg/dL).
[c]Bone lesions: one or more osteolytic lesions on skeletal radiography, computed tomography, or positron emission tomography–computed tomography.
[d]$p<0.01$, statistically significant.

Table 3. Univariate and multivariate analyses of factors associated with DS stage of MM

Clinicopathological parameters	Univariate analysis		Multivariate analysis	
	HR (95% CI)	p value	HR (95% CI)	p value
β_2-Microglobulin (<3.5 μg/mL vs. ≥3.5 μg/mL)	3.992 (1.513–10.529)	0.005[a]	5.404 (1.392–20.975)	0.015[b]
M-protein (IgA vs. IgG)	0.531 (0.180–1.563)	0.251	—	—
Light chain (λ vs. κ)	0.577 (0.237–1.405)	0.226	—	—
Hemoglobin, g/L (<120 g/L vs. ≥120 gL)	0.529 (0.196–1.432)	0.210	—	—
Total protein (abnormal value vs. normal value)	2.333 (0.954–5.708)	0.063	—	—
Albumin (abnormal value vs. normal value)	2.600 (1.052–6.424)	0.038[b]	—	—
Globulin (abnormal value vs. normal value)	2.333 (0.954–5.708)	0.063	—	—
Renal injury (yes vs. no)	3.117 (1.206–8.056)	0.019[b]	—	—
Bone injury (yes vs. no)	13.440 (4.487–40.253)	<0.001[b]	16.706 (4.241–65.798)	0.001[a]
TUG1 (low expression vs. high expression)	21.700 (4.772–97.773)	<0.001[c]	19.607 (3.630–105.893)	0.001[a]

Univariate and multivariate analyses were carried out using a logistic regression analysis approach.
CI=Confidence interval; *HR*=hazard rate.
[a]$p < 0.01$, statistically significant.
[b]$p < 0.05$, statistically significant.
[c]$p < 0.001$, statistically significant.

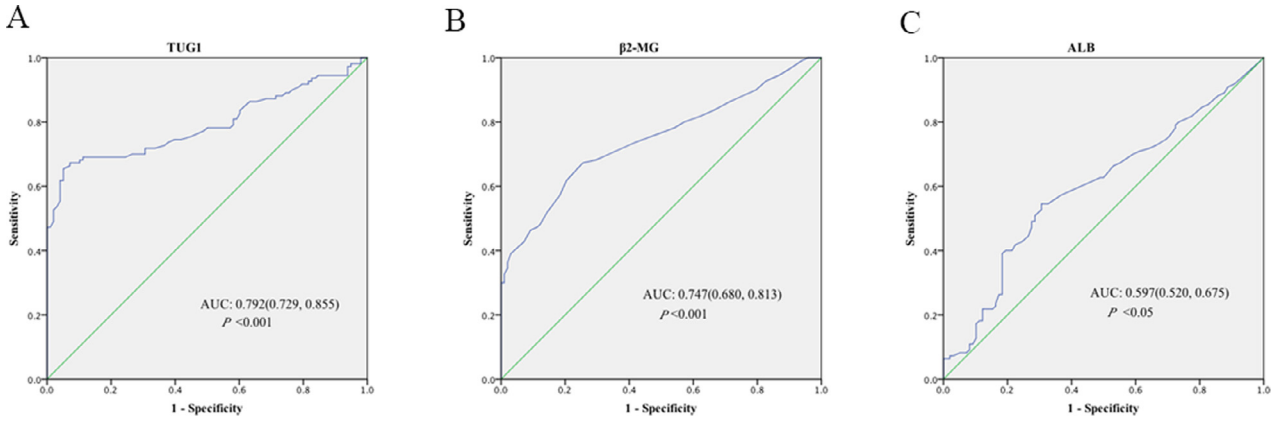

Figure 4. Receiver operating characteristic curves for serum TUG1 **(A)**, β_2-MG **(B)**, and ALB **(C)** for MM diagnosis.

Table 4. ROC curves for a combination of TUG1, β_2-MG, and ALB as a means of discriminating patients with MM and healthy controls in series mode

	SEN	SPE	ACCU	PPV	NPV	AUC (95% CI)
β_2-MG	65.5% (72/110)	79.6% (78/98)	72.1% (150/208)	78.3% (72/92)	67.2% (78/116)	0.747 (0.680−0.813)
ALB	54.5% (60/110)	69.4% (68/98)	58.6% (122/208)	66.7% (60/90)	57.6% (68/118)	0.597 (0.520−0.675)
TUG1	65.5% (72/110)	94.9% (93/98)	79.3% (165/208)	93.5% (72/77)	71.0% (93/131)	0.792 (0.729−0.855)
TUG1+ β_2-MG	71.8% (79/110)	96.9% (95/98)	83.7% (164/208)	96.3% (79/82)	75.4% (95/126)	0.836 (0.779−0.894)
TUG1+ ALB	65.5% (72/110)	94.9% (93/98)	79.3% (165/208)	93.5% (72/77)	71.0% (93/131)	0.791 (0.728−0.855)
TUG1+ β_2-MG+ ALB	71.8% (79/110)	96.9% (95/98)	83.7% (164/208)	96.3% (79/82)	75.4% (95/126)	0.836 (0.779−0.894)

ACCU=Overall accuracy; *NPV*=negative predictive value; *PPV*=positive predictive value; *SEN*=sensitivity; *SPE*=specificity.

results suggest that serum lncRNAs have the potential to be valuable biomarkers useful for diagnosing cancer and determining patient prognosis.

TUG1 is an lncRNA that was recently determined to be dysregulated in a range of cancers, wherein it has been reported to play a range of distinct biological roles. For example, Isin et al. first explored the relevance of TUG1 in MM [35]. They analyzed five different lncRNAs (TUG1, LincRNA-p21, MALAT1, HOTAIR, and GAS5), finding LincRNA-p21 to be significantly differentially expressed only in patients with CLL, whereas the other lncRNAs were significantly differentially expressed in those with MM, with only TUG1 levels being significantly higher than in healthy controls. The specific association between levels of TUG1 and patient clinicopathological findings, however, remained uncertain.

In this study, we confirmed that TUG1 levels were detectable in the serum of MM patients at higher levels than in controls, beginning by validating these results in 20 MM patients. We further confirmed that this lncRNA was upregulated in both MM cell lines and bone marrow tissue samples as compared with appropriate normal controls, leading us to conclude that TUG1 expression is significantly increased in MM patients. We then expanded these analyses to a larger patient cohort, revealing qRT-PCR to allow for reliable, reproducible, sensitive, and linear detection of serum TUG1 in serum samples. This larger analysis further confirmed that TUG1 levels were increased in those with MM, suggesting TUG1 may be a valuable diagnostic biomarker of MM. We then determined that serum TUG1 levels correlated with MM clinical stage and disease severity, as well as with levels of other diagnostic markers of MM including total protein, ALB, and β_2-MG. Individuals with bone injuries also exhibited higher serum TUG1 levels than those without such injuries ($p = 0.007$). As bone injury is a key symptom of MM progression, this suggests that TUG1 may be associated with MM development.

We further employed univariate and multivariate analyses to explore the diagnostic utility of TUG1, comparing TUG1 expression levels and disease stage in MM patients. These analyses suggested that TUG1 may independently predict DS stage in MM patients. Using ROC analysis, we found TUG1 to have an AUC of 0.792 (95% CI: 0.729−0.855, $p < 0.001$) with a sensitivity and specificity of 65.5% and 94.9%, respectively, which were superior to these values for ALB (54.5%, 69.4%) and β_2-MG (65.5%, 79.6%), indicating that TUG1 levels may be a valuable diagnostic biomarker of MM. However, this study included only a limited number of patients. When we combined all three of these biomarkers, we found that TUG1, ALB,

and β_2-MG together significantly enhanced the AUC to 0.836 (95% CI: 0.779-0.894, $p < 0.001$), which was better than for any individual marker, suggesting that TUG1 should be used together with these other diagnostic indicators.

In summary, our results reveal that MM patients exhibit significantly increased serum TUG1 levels, with lncRNA representing a potentially valuable diagnostic biomarker suitable for clinical utilization. This biomarker has the potentially to be used for auxiliary diagnosis of MM either alone or together with other diagnostic indicators. Future research should focus on exploring the relevance of TUG1 in a larger cohort, in a longitudinal manner, and in the context of chemotherapeutic responses. In addition, it remains uncertain as to what if any physiological role is played by TUG1 in MM, and as such further studies will focus on the mechanistic role of TUG1 in this disease.

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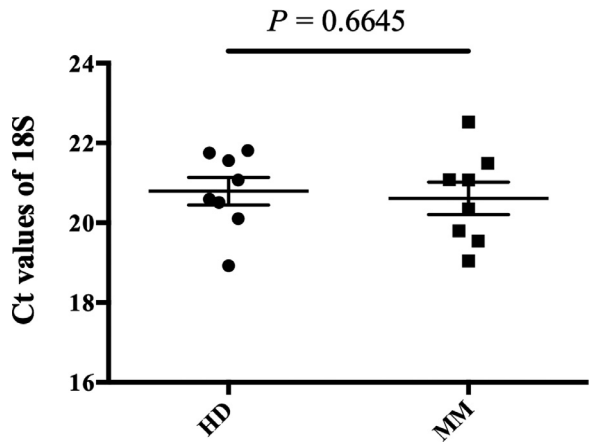
Supplementary Table E1. Name, primer sequences, and product size of candidate reference genes

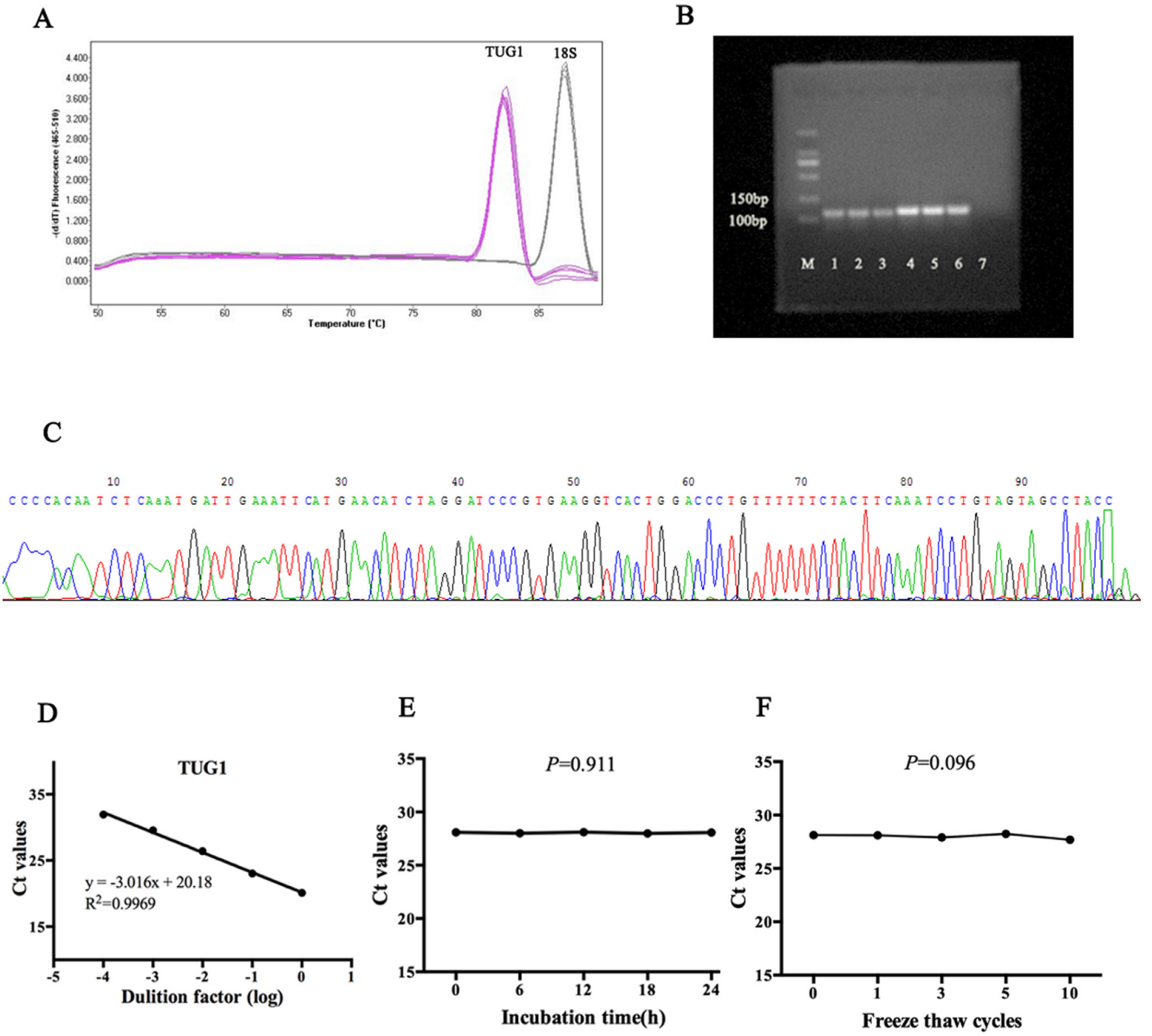
Gene symbol	Gene name	Primer sequences (forward/reverse)	Product length (bp)
18S	18S ribosomal RNA	5'-GTAACCCGTTGAACCCCATT-3' 5'-CCATCCAATCGGTAGTAGCG-3'	151
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	5'-CGCTCTCTGCTCCTCCTGTTC-3' 5'-ATCCGTTGACTCCGACCTTCAC-3'	108
ACTB	β-actin	5'-TTGTTACAGGAAGTCCCTTGCC-3' 5'-ATGCTATCACCTCCCCTGTGTG-3'	101
RPII	polymerase (RNA) II	5'-GCACCACGTCCAATGACAT-3' 5'-GTGCGGCTGCTTCCATAA-3'	267
TUB	α-tubulin	5'-CTTGGAACCCACAGTCATT-3' 5'-CCCTCGGGCATAGTTATT-3'	112
TUG1	taurine upregulated gene 1	5'-CTGAAGAAAGGCAACATC-3' 5'-GTAGGCTACTACAGGATTTG-3'	141

Supplementary Table E2. Ct values of 5 candidate reference genes

Reference	Mean ± SD	CV (%)
18S	21.51±0.9786	4.55%
GAPDH	25.79±0.7124	2.76%
TUB	28.68±0.9017	3.14%
ACTB	22.90±1.0010	4.37%
RPII	32.79±0.4884	1.49%



Supplementary Figure E1. Ct values of 18 S rRNA in healthy donors ($n = 8$) and MM patients ($n = 8$).



Supplementary Figure E2. Methodological assessment of lncRNA TUG1 detection. **(A)** TUG1 and 18 S rRNA melting peaks. **(B)** Agarose gel electrophoresis PCR products: (M) 500-bp DNA marker, (1–3) lncRNA TUG1 PCR products; (4–6) 18 S PCR products; (7) negative control. **(C)** Confirmation of lncRNA TUG1 via Sanger sequencing. **(D)** TUG1 detection linearity as assessed with 10-fold serial dilutions. **(E, F)** TUG1 stability under harsh environmental conditions.