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# Long non-coding RNA taurine upregulated 1 regulates the progression of head and neck cancer

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## ABSTRACT

Taurine upregulated 1 (TUG1), whose function is associated with tumor development, is a relatively new long non-coding RNA. TUG1 is overexpressed in multiple types of cancers. However, in head and neck squamous cell carcinoma (HNSCC), the behavior of TUG1 has not yet been completely elucidated. Therefore, we aimed to clarify the function of TUG1 in HNSCC and develop a novel therapeutic target. We analyzed the expression levels of TUG1 in patients with HNSCC using The Cancer Genome Atlas dataset and human oral keratinocytes, and five HNSCC cell lines (HSC-4, Sa3, HSQ-89, SAS, and Ca9-22) through quantitative reverse-transcription polymerase chain reaction. The biological role of TUG1 in HNSCC was investigated using cell growth and migration assays with antisense oligonucleotides in Ca9-22 and SAS cell lines. TUG1 target genes were identified via microarray analysis. The TUG1 expression level was considerably higher in tumor than in normal tissues, and the same result was observed in human oral keratinocytes and all HNSCC cell lines. TUG1 knockdown dramatically inhibited cell proliferation and migration. Furthermore, we identified nemo-like kinase, which may change in tandem with TUG1 expression. Our findings indicate the possibility for targeting the TUG1-nemo-like kinase axis as a novel approach for the treatment of HNSCC.

Keywords: long non-coding RNA, head and neck cancer, taurine upregulated gene1

Abbreviations:
ASO: antisense oligonucleotides
HNSCC: head and neck squamous cell carcinoma
lncRNA: long non-coding RNA
NLK: nemo-like kinase
TUG1: taurine upregulated 1



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## INTRODUCTION

Head and neck squamous cell cancer (HNSCC) is one of the poorest prognostic malignant tumors because of its high metastasis and recurrent rates.[1] Surgery remains the standard treatment; however, postoperative functional and esthetic disabilities are major problems. Furthermore, despite recent advances in therapeutic interventions, the 5-year survival rate of HNSCC patients has not improved. Therefore, clarifying the molecular mechanisms underlying HNSCC and identifying therapeutic targets are urgent clinical issues worldwide.

According to previous reports,[2-3] apparent driver gene mutations have not been identified in HNSCC; therefore, epigenetic modifications that regulate gene expression at both transcriptional and post-transcriptional levels may play a crucial role in this type of cancer. Long non-coding RNAs (lncRNAs) are sequences of more than 200 nucleotides in length that have no protein-coding capacity.[4] lncRNAs are pivotal regulators of diverse biological processes and may contribute to cancer development as oncogenes or tumor suppressors.[5] These studies suggest that lncRNAs play context-dependent roles in different cancer types. The dysregulation of lncRNAs is associated with cancer development.

Taurine-upregulated 1 (TUG1) is a relatively new lncRNA and is considerably associated with tumor development by regulating cell proliferation, invasion, metastasis, and apoptosis, and drug resistance.[6,7] TUG1 is overexpressed in various types of cancers including gastric,[8] bladder,[9] and hepatocellular cancers.[10] However, the biological functions of TUG1 in HNSCC have not yet been completely elucidated. The purpose of this study was to clarify the function of TUG1 in HNSCC and develop a novel therapeutic target.

In the current study, we showed that TUG1 was substantially upregulated in HNSCC, and that TUG1 knockdown inhibited cell growth and migration. Furthermore, microarray analysis revealed that the expression of TUG1 was correlated with that of nemo-like kinase (NLK), a gene that encodes a serine/threonine protein kinase and is involved in human carcinogenesis. Our observations indicate that the TUG1-NLK axis may be an effective epigenetic target for the treatment of HNSCC.

## MATERIALS AND METHODS

*Cell culture*

Five HNSCC cell lines, HSC-4, Sa3, HSQ-89, SAS, and Ca9-22, were purchased from Riken BRC (Tsukuba, Japan) and cultured in Dulbecco's modified Eagle's medium (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA) and an antibiotic-antimycotic reagent (Wako). Cells were cultured at 37 °C in a humidified incubator with 5% $CO_2$.

*RNA extraction and quantitative reverse-transcription polymerase chain reaction analysis*

Total RNA was isolated from five HNSCC cell lines using TRIzol reagent (Thermo Fisher Scientific) according to manufacturer's protocol. Total RNA derived from primary human oral keratinocytes was purchased from Scientific Cell Research Laboratories (Carlsbad, CA, USA). Total RNA (2 μg) was converted to cDNA using a PrimeScript RT Master Mix (Takara Bio Inc, Tokyo, Japan). Quantitative polymerase chain reaction (qPCR) was performed using the Mx3005P Real-Time QPCR System (Agilent Technologies, Santa Clara, CA, USA). SYBR Green qPCR was performed on the target genes in triplicates. The primer sets used for the qPCR assay are listed in Table 1.

**Table 1** Primer sets for qRT-PCR analysis and the sequence for ASO

| ***qRT-PCR*** | ***Primer sequence (5′ to 3′)*** |
|---|---|
| *Target gene* | |
| *TUG1* | *Forward:* AGGTAGAACCTCTATGCATTTTGTG |
| *(SYBR)* | *Reverse:* ACTCTTGCTTCACTACTTCATCCAG |
| *NLK* | *Forward:* ATCATCAGCACTCGCATCATC |
| *(SYBR)* | *Reverse:* GACCAGACAACACCAAAGGC |
| *GAPDH* | *Forward:* GACCAGACAACACCAAAGGC |
| *(SYBR)* | *Reverse:* CCCCACTTGATTTTGGAGGGA |
| ***ASO sequence*** | ***Sequence (5′ to 3′)*** |
| **TUG1 #1* | *T(L)G(L)A(L)A(L)tttcaatcatttgA(L)G(L)A(L)T(L)* |
| **TUG1 #2* | *T(L)T(L)A(L)5(L)tctgggcttctG(L)5(L)A(L)5(L)* |
| **Control* | *T(L)C(L)G(L)aagtactcagcgtaaG(L)T(L)T(L)* |

*Lower case: DNA N(L):LNA 5(L):LNA-mC
ASO: antisense oligonucleotides
GAPDH: glyceraldehyde-3-phosphate dehydrogenase
NLK: nemo-like kinase
qRT-PCR: quantitative reverse-transcription polymerase chain reaction
TUG1: taurine upregulated 1

*Antisense oligonucleotide-mediated knockdown of TUG1*

To downregulate gene expression with antisense oligonucleotides (ASO), HNSCC cell lines were transfected with 10 nM ASO targeting TUG1 or control non-targeting (CTRL) ASO using Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer's instructions. Total RNA was extracted from the cell lines 24 hours after the transfection of ASOs and the effects were validated. The ASO sequences are listed in Table 1.

*Cell proliferation and migration assays*

For the quantitative analysis of cell proliferation, cells were seeded in 96-well plates and cultured for 24 hours before ASO treatment. Afterward, 10 μL of WST-8 solution (Cell Counting Kit-8, Dojindo, Kumamoto, Japan) was added to each well and incubated at 37 °C for 1 hour in a humidified $CO_2$ incubator. The absorbance was monitored at 450 nm using a microplate reader (SmartSpec3000, BIO-RAD, Tokyo, Japan).

For the quantitative analysis of cell migration, after 48 hours of ASO treatment cells were seeded in 12-well plates and cultured to create a confluent monolayer. A wound healing assay was performed on the monolayer cells using a p1000 pipette tip. Images were captured at 0 hour (immediately after scratching) and 24 hours after incubation. The area without cells was measured using the ImageJ software. The migration rate was calculated as (the area without cells at 0 hour minus that at 24 hours) divided by (the area without cells at 0 hour).

*Microarray analysis*

For microarray analysis, Ca9-22 cell line was treated with either control ASO or TUG1 ASO #1 and #2 for 24 hours. Total RNA was isolated using TRIzol reagent and shipped to Filgen Inc (Aichi, Japan) for GeneChip Arrays.

*Gene expression and clinicopathological data for HNSCC patients*

Gene expression and clinicopathological data for patients with HNSCC in The Cancer Genome Atlas were obtained from the UCSC Xena database (https://xena.ucsc.edu/, accessed May 23, 2023).

*Statistical analysis of clinical features associations*

We used GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA) for statistical analysis and the statistical significance of the differences between two groups was analyzed using a paired Student's t-test. All reported P values were two-sided, with $P < 0.05$ taken as statistically significant.

## RESULTS

*TUG1 expression was considerably higher in tumor than in normal tissue, both in clinical samples and cell lines*

First, we examined the expression level of TUG1 in the HNSCC cohort using The Cancer Genome Atlas dataset. TUG1 expression was significantly higher in tumor samples (n = 500) than in normal samples (n = 44; Fig. 1A). Furthermore, compared with samples from the same patients (n = 43), TUG1 expression was significantly higher in tumors than in the corresponding

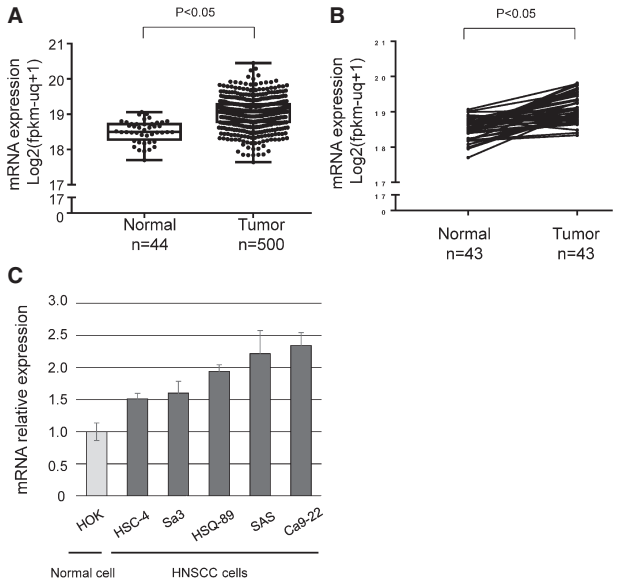


**Fig. 1** TUG1 expression level in HNSCC samples and cell lines

**Fig. 1A:** TUG1 expression level in normal (n = 44) and tumor (n = 500) tissues in the HNSCC samples. The median is shown by a thick line inside the box, the ends of which indicate the upper and lower quartiles. Error bars indicate the minimum to maximum and show all points.

**Fig. 1B:** TUG1 expression level in corresponding adjacent normal and tumor tissues in the same patients (n = 43).

**Fig. 1C:** TUG1 expression level in the five HNSCC cell lines (HSC-4, Sa3, HSQ-89, SAS, Ca9-22) and primary human oral keratinocytes (HOK). mRNA expression level was normalized to GAPDH mRNA level and indicated in the y-axis (left). The error bars indicate standard deviation. *P < 0.05.

GAPDH: glyceraldehyde-3-phosphate dehydrogenase
HNSCC: head and neck squamous cell carcinoma
mRNA: messenger RNA
TUG1: taurine upregulated 1

adjacent normal samples (Fig. 1B). We also examined TUG1 expression via quantitative reverse-transcription PCR in five HNSCC cancer cell lines (HSC-4, Sa3, HSQ-89, SAS, and Ca9-22) and primary human oral keratinocytes. All five HNSCC cell lines showed significantly upregulated TUG1 expression compared to that in human oral keratinocytes (Fig. 1C). These results indicate that TUG1 is upregulated in HNSCC tumors compared to adjacent normal tissue.

*Knockdown of TUG1 expression inhibited cell growth and migration in HNSCC cell lines*

To clarify the biological role of TUG1 in HNSCC, we examined the effects of TUG1 inhibition using cell growth and migration assays in Ca9-22 and SAS cell lines, which show the highest

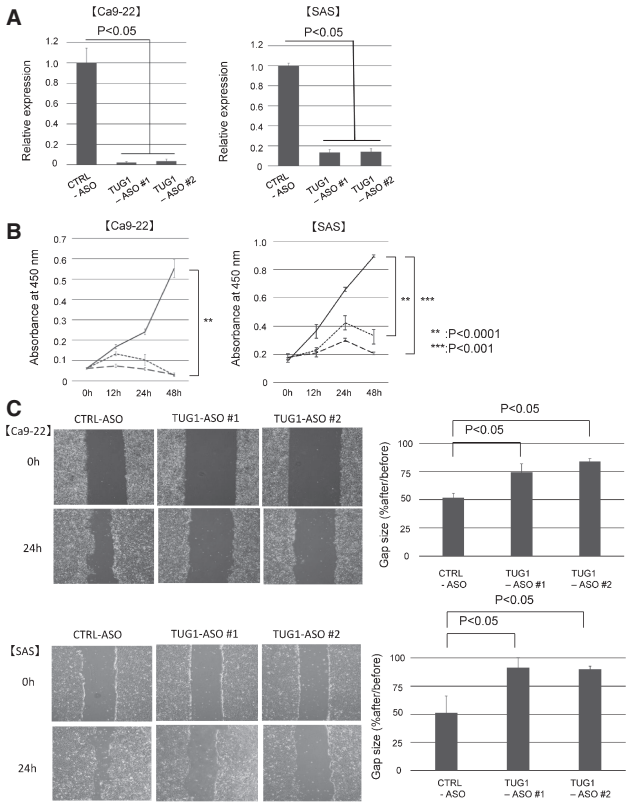


**Fig. 2** Effect of TUG1 on HNSCC cell proliferation and migration

**Fig. 2A:** TUG1 expression level in SAS and Ca9-22 cell lines 24 hours after transfection of negative control (CTRL) ASO and TUG1 ASO #1 and #2. mRNA expression level was normalized to GAPDH mRNA level and indicated in the y-axis (left). The error bars indicate standard deviation. *P < 0.05.

**Fig. 2B:** Effect of TUG1 ASO on SAS and Ca9-22 cells proliferation. The y-axis indicates the absorbance at the wavelength of 450 nm. The error bars indicate standard deviation. *P < 0.05.

**Fig. 2C:** Effect of TUG1 ASO on SAS and Ca9-22 cells migration. The images were captured at 0 hour and 24 hours. The black lines show the area without cells. SAS and Ca9-22 cell migration rates were calculated and are shown. The error bars indicate standard deviation. *P < 0.05.

ASO: antisense oligonucleotides
GAPDH: glyceraldehyde-3-phosphate dehydrogenase
HNSCC: head and neck squamous cell carcinoma
mRNA: messenger RNA
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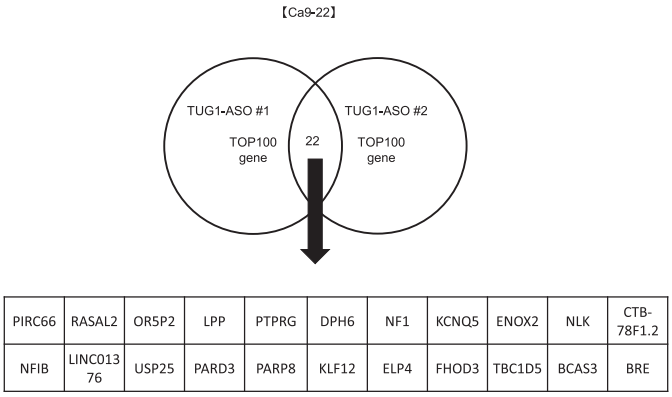
expression levels of TUG1. Two different ASO (TUG1-ASO #1/ #2) were designed to knockdown TUG1 expression, and as shown in Fig. 2A, TUG1 expression in both Ca9-22 and SAS cells was markedly reduced compared to the control ASO. Cell growth was significantly suppressed by TUG1 depletion in both Ca9-22 and SAS cell lines ($P < 0.05$) (Fig. 2B). A scratch assay was performed to assess the effects of TUG1 on HNSCC cell migration. Depletion of TUG1 expression also significantly decreased the migration of Ca9-22 and SAS cell lines ($P < 0.05$) (Fig. 2C). These data indicated that TUG1 exerts its oncogenic activity in HNSCC via multiple processes.

*Microarray analysis*

To identify the genes whose expression changed in tandem with TUG1 expression, we performed gene expression microarray analysis in Ca9-22 cell line treated with CTRL-ASO, TUG1-ASO #1, and TUG1-ASO #2. We extracted the top 100 genes whose expression was reduced by more than two-fold compared with that of CTRL-ASO cells. This analysis revealed that 22 genes were commonly downregulated in both TUG1-ASO#1 and #2 compared with CTRL -ASO. Among these 22 genes (Fig. 3), we focused on NLK, an evolutionarily conserved mitogen-activated serine/threonine protein kinase whose expression is known to be closely related to the carcinogenesis of human cancers.




| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| PIRC66 | RASAL2 | OR5P2 | LPP | PTPRG | DPH6 | NF1 | KCNQ5 | ENOX2 | NLK | CTB-78F1.2 |
| NFIB | LINC01376 | USP25 | PARD3 | PARP8 | KLF12 | ELP4 | FHOD3 | TBC1D5 | BCAS3 | BRE |

**Fig. 3** Genes whose expression was changed in tandem with TUG1 expression
The top 100 genes whose expression was downregulated by TUG1-ASO #1 and #2 compared to CTRL-ASO in Ca9-22 cells were extracted. The Venn diagram shows the number of genes that were downregulated in both TUG1-ASO #1 and #2 compared to CTRL-ASO in Ca9-22 cells.
ASO: antisense oligonucleotides
CTRL: control non-targeting
TUG1: taurine upregulated 1

*TUG1 expression is closely associated with NLK expression*

We examined the expression level of NLK in an HNSCC cohort using The Cancer Genome Atlas dataset. In addition to TUG1, NLK expression was significantly higher in tumor (n = 500) than in normal samples (n = 44; Fig. 4A). We confirmed that NLK expression was efficiently reduced in both Ca9-22 and SAS cell lines following treatment with TUG1 ASO #1 (Fig. 4B). Furthermore, tumor samples with high TUG1 expression showed significantly upregulated NLK expression compared to those with low TUG1 expression (Fig. 4C). NLK expression positively correlated with TUG1 expression in tumor (Fig. 4D). These data suggested that TUG1 is closely associated with NLK expression in HNSCCs cells.

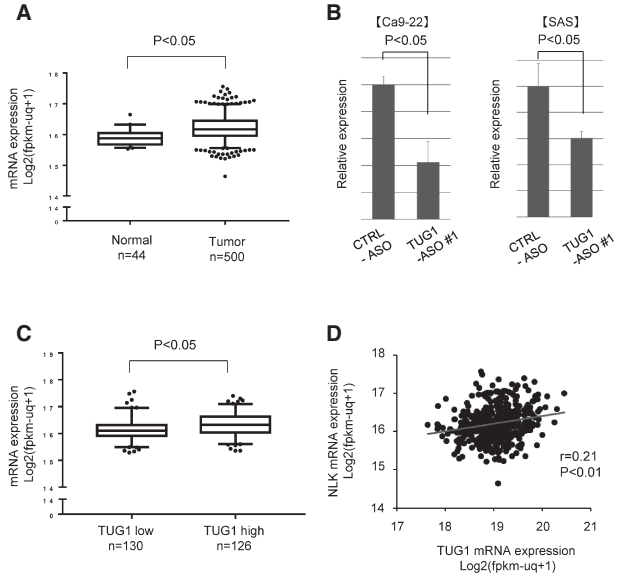



**Fig. 4** Correlation between TUG1 and NLK

**Fig. 4A:** NLK expression level in normal (n = 44) and tumor (n = 500) tissues in the HNSCC samples. The median is shown by a thick line inside the box, the ends of which indicate the upper and lower quartiles. Error bars represent the 5 and 95 percentile values and show all points.

**Fig. 4B:** Expression level of NLK in Ca9-22 and SAS cell lines 24 hours after transfection of negative control (CTRL) ASO and TUG1 ASO #1. mRNA expression level was normalized to GAPDH mRNA level and indicated in the y-axis (left). The error bars indicate standard deviation. *P < 0.05.

**Fig. 4C:** Expression level of NLK in low (n = 130) and high (n = 126) expression of TUG1 in HNSCC samples. The median is shown by a thick line inside the box, the ends of which indicate the upper and lower quartiles. Error bars represent the 5 and 95 percentile values and show all points.

**Fig. 4D:** Scatter plot showing Pearson's correlation between TUG1 and NLK expression.

ASO: antisense oligonucleotides
GAPDH: glyceraldehyde-3-phosphate dehydrogenase
HNSCC: head and neck squamous cell carcinoma
mRNA: messenger RNA
NLK: nemo-like kinase
TUG1: taurine upregulated 1

## DISCUSSION

lncRNAs play crucial roles in cancer cells development, progression, migration, and invasion.[11] In HNSCC, several lncRNAs, including hox transcript antisense intergenic RNA (HOTAIR), nuclear enrich abundant transcript 1 (NEAT1), and metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), have been identified as oncogenic regulators.[12-14] These reports suggest that lncRNAs can be used as therapeutic targets in HNSCC.

TUG1 acts as an oncogenic regulator in several types of cancers; however, whether it could be a therapeutic target for treating HNSCC remains unclear. In HNSCC, TUG1 positively controls the Wnt/β-catenin signaling pathway and promotes proliferation and migration of cancer cells.[15] In addition, inhibition of the Wnt/β-catenin signaling pathway increases sensitivity of cancer cells to 5-fluorouracil,[16] whereas overexpression of β-catenin induces cisplatin resistance.[17] Therefore, inhibition of TUG1 may enhance antitumor effects of cytotoxic anticancer drugs.

In the current study, we confirmed that TUG1 expression was considerably higher in HNSCC tissues than in normal tissues. The same results were also observed in HNSCC cell lines. In vitro functional studies showed that TUG1 knockdown markedly suppressed cell growth and migration. These findings suggest that TUG1 contributes to the tumorigenesis of HNSCC and may play an oncogenic role. Comprehensive gene expression analysis using microarray revealed that the expression of TUG1 was similar to that of several other genes. Among these, we focused on *NLK*, because this gene regulates tumor growth in oral squamous cell carcinoma, a subtype of HNSCC.[18] Moreover, *NLK* is involved in cisplatin sensitivity under TUG1 regulation.[19] This gene is related to mitogen-activated protein kinases and is highly conserved during evolution. This gene is widely expressed in many tissues and organs of the body,[20] and is known to regulate cancer transcription factors and plays pivotal roles in cell proliferation, invasion, and apoptosis.[21] NLK also plays context-dependent roles in various types of cancer. However, little is known about its function in HNSCC.

In this study, we showed that NLK was substantially overexpressed in HNSCC tissues compared to normal tissues, suggesting that NLK might participate in the regulation of HNSCC. Furthermore, a positive correlation was observed between TUG1 and NLK expression, and TUG1 knockdown inhibited NLK expression. These results suggest that TUG1 has a potential role in the regulation of NLK expression. However, this study had some limitations. We did not assess whether TUG1 and NLK were directly bound to each other or indirectly regulated by small molecules. TUG1 contains microRNA (miRNA)-binding sites and may act as a miRNA sponge.[5] For example, one study reported that TUG1 regulates oral squamous cell carcinoma development through miR-524-5p, which mediates distal-less homeobox 1 (DLX1) expression.[22] Another group reported that TUG1 promotes the progression of oral squamous cell carcinoma by formin like 2 (FMNL2) upregulation through sponging miR-219.[23] Further studies are needed to elucidate the mediating molecular mechanisms about the TUG1-NLK axis. Our findings indicate the possibility for targeting the TUG1-NLK axis as a novel approach for the treatment of HNSCC.

## CONFLICT OF INTEREST DISCLOSURE

The authors declare no conflicts of interest.

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