Glutathione S-transferase P1 (GSTP1) suppresses cell apoptosis and its regulation by *miR-133a* in head and neck squamous cell carcinoma (HNSCC)

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Abstract. The glutathione S-transferase P1 (GSTP1) protein plays several critical roles in both normal and neoplastic cells, including phase II xenobiotic metabolism, stress responses, signaling and apoptosis. Overexpression of *GSTP1* has been observed in many types of cancer, including head and neck squamous cell carcinoma (HNSCC). However, the role of *GSTP1* in HNSCC is not well understood. We investigated the role of *GSTP1* in two HNSCC cell lines, HSC3 and SAS. Silencing of *GSTP1* revealed that cancer cell proliferation was significantly decreased in both cell lines. In addition, the frequency of apoptotic cells increased following si-*GSTP1* transfection of HSC3 and SAS cell lines. Growing evidence suggests that microRNAs (miRNAs) negatively regulate gene expression and can function as oncogenes or tumor suppressors in human cancer. Based on the results of web-based searches, *miR-133a* is a candidate miRNA targeting *GSTP1*. Down-regulation of *miR-133a* has been reported in many types of human cancer, including HNSCC. Transient transfection of *miR-133a* repressed the expression of *GSTP1* at both the mRNA and protein levels. The signal from a luciferase reporter was significantly decreased at one *miR-133a* target site at the 3'UTR of *GSTP1*, suggesting that *miR-133a* directly regulates *GSTP1*. Our data indicate that *GSTP1* may have an oncogenic function and may be regulated by *miR-133a*, a tumor suppressive miRNA in HNSCC. The identification of a novel oncogenic pathway could provide new insights into potential mechanisms of HNSCC carcinogenesis.

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Introduction

Head and neck squamous cell carcinoma (HNSCC) constitutes the sixth most common malignancy worldwide (1). In spite of considerable advances in surgery, radiotherapy and chemotherapy, the overall five-year survival rate for patients with this type of cancer is among the lowest of all major cancer types and has not improved dramatically during the last decade (2). Local tumor recurrence and distant metastasis after conventional therapy appear to be major contributing factors decreasing survival of HNSCC patients. Therefore, understanding the molecular oncogenic pathways underlying HNSCC would be helpful to improve diagnosis, therapy, and prevention of the disease.

Glutathione S-transferase P1 (GSTP1), a member of the GST enzyme superfamily, catalyzes the conjugation of electrophiles to glutathione in the process of detoxification (3). Recently, we demonstrated that *GSTP1* is highly expressed in human HNSCC tissue (4) and is frequently overexpressed in many other cancers, including tumors of the brain, breast, ovary, esophagus, stomach, pancreas, colon, skin, kidney, lung, bile ducts, bladder and lymphatic and hematopoietic systems (5-10). As such, there has been considerable clinical interest in *GSTP1* as a tumor marker and as a therapeutic target (11,12). However, little is known about the functional role of *GSTP1* in HNSCC.

microRNAs (miRNAs), are endogenous small non-coding RNAs (19-22 nucleotides) that can control gene expression by targeting mRNAs for cleavage or translational repression (13). Bioinformatic predictions indicate that miRNAs regulate more than 30% of the protein coding genes (14). It is estimated that approximately 1,000 miRNAs exist in the vertebrate genome. Release 16.0 of the miRBase database (http://microrna.sanger.ac.uk/) included 1,048 registered human miRNAs. Growing evidence suggests that miRNAs are involved in several crucial biological processes, including development, differentiation, apoptosis, and proliferation (13,15). Furthermore, miRNAs can function either as oncogenes or tumor suppressors and are aberrantly expressed in several types of human cancer (16-18).

A search of the TargetScanHuman release 5.1 database, identified *miR-133a* as a candidate miRNA regulator of *GSTP1*. Down-regulation of *miR-133a* has been reported in many types of human cancer, including HNSCC (19-22). More recently, we demonstrated that *miR-133a* functions as a tumor suppressor in esophageal cancer and bladder cancer (23,24). In this study, we conducted a functional analysis of *GSTP1* and investigated whether *GSTP1* is directly regulated by *miR-133a* in HNSCC cell lines. The identification of a novel oncogenic pathway could provide new insights into potential mechanisms of HNSCC carcinogenesis.

Materials and methods

Clinical HNSCC specimens. Twenty pairs of HNSCC (oral cavity, n=9; larynx, n=3; oropharynx, n=3; hypopharynx n=5) and corresponding normal epithelial samples were obtained from patients at the Chiba University Hospital (Chiba, Japan) from 2007 to 2009. All tissue specimens were obtained from patients undergoing surgical treatment. Normal tissues were obtained far from the center of the cancer. No cancer cells were detected in neighboring formalin-fixed, paraffin-embedded tissues. Written consent of tissue donation for research purposes was obtained from each patient before tissue collection. The protocol was approved by the Institutional Review Board of Chiba University. The specimens were immersed in RNA*later* (Qiagen, Valencia, CA, USA) and stored at -20˚C until RNA was extracted.

HNSCC cell culture. Human HNSCC cell lines (HSC3 and SAS) were provided by the American Type Culture Collection (ATCC, Manassas, VA, USA). Both cell lines were grown in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F-12) supplemented with 10% fetal bovine serum in a humidified atmosphere containing 5% CO_2 at 37˚C. HSC3 was derived from a lymph node in tongue squamous cell carcinoma, while SAS came from a primary region of tongue squamous cell carcinoma.

RNA isolation. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. RNA concentrations were determined spectrophotometrically, and the molecule integrity was checked by gel electrophoresis. RNA quality was confirmed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

Real-time quantitative RT-PCR. First-strand cDNA was synthesized from 1 μg total RNA using random primers and the Reverse Transcription (RT) system (Promega, Tokyo, Japan). Gene-specific PCR products were continuously assayed using a 7900-HT real-time PCR System according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA). The initial PCR step consisted of a 10 min hold at 95˚C, followed by 40 cycles consisting of a 15 sec denaturation at 95˚C and a 1 min annealing/extension at 63˚C. TaqMan® probes and primers for *GSTP1* (P/N: Hs00168310_m1) and the *GAPDH* (A/N: NM_002046) internal control were obtained from Applied Biosystems (Assay-On-Demand gene expression products). The expression levels of *miR-133a* (P/N: 002246) were analyzed by TaqMan quantitative real-time PCR (TaqMan® MicroRNA Assay; Applied Biosystems) and normalized to *RNU48* (A/N: X96648). All reactions were performed in triplicate, and included negative control reactions that lacked cDNA.

Immunoblotting and immunohistochemistry. Cells were harvested 72 h after transfection and lysates were prepared. Fifty μg of protein from cell lysates were separated by NuPAGE on 4-12% Bis-Tris gels (Invitrogen) and transferred to PVDF membranes. Immunoblotting was performed with diluted (1:100) monoclonal *GSTP1* antibody (ab47709, Abcam, Cambridge, UK). The β-actin antibody (sc-1615; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as an internal control. The membrane was washed and incubated with goat anti-mouse IgG (H+L)-HRP conjugate (Bio-Rad, Hercules, CA, USA). Specific complexes were visualized by echochemiluminescence (GE Healthcare Bio-Sciences, Princeton, NJ, USA).

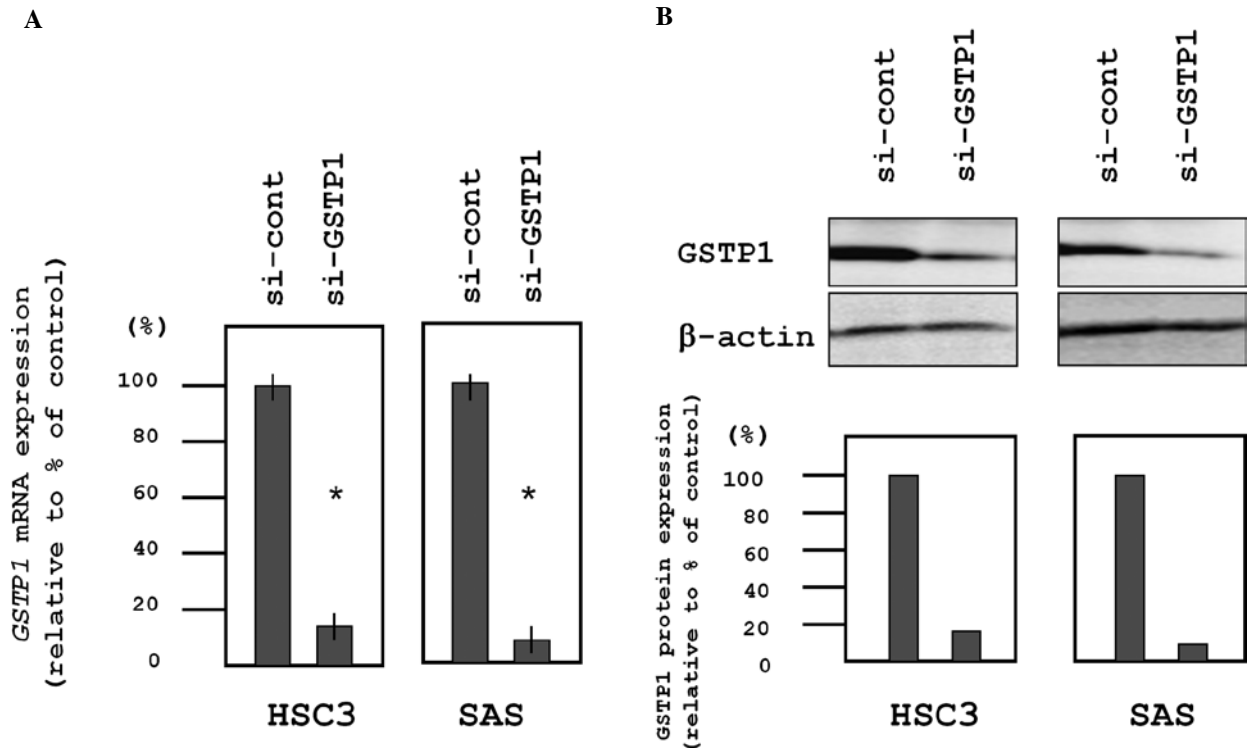
For immunohistochemistry, primary mouse monoclonal antibodies against *GSTP1* (Cell Signaling) were diluted 1:200. The slides were treated with biotinylated anti-mouse IgG (H+L) made in house (Vector Laboratories, Burlingame, CA, USA). Diaminobenzidine-hydrogen peroxide (Sigma-Aldrich, St. Louis, MO, USA) was the chromogen, and counterstaining was carried out with 0.5% hematoxylin.

si-GSTP1 and mature miRNA transfection in HNSCC cell lines. As described previously (24,25), si-*GSTP1* and si-control molecules were transfected into the HNSCC cell lines with Opti-MEM (Invitrogen) and Lipofectamine™ RNAiMax reagent (Invitrogen). After co-transfecting 10 nM small interfering RNA, si-*GSTP1* (P/N: s194476; Applied Biosystems) or si-control (D-001810-10; Thermo Fisher Scientific), HNSCC cells were seeded into 96-well plates at $3x10^3$ cells per well for proliferation assays or 6-well plates at $3x10^4$ for apoptosis assays.

Mature miRNA molecules, Pre-miR™ miRNA precursors, and a negative control (Applied Biosystems, Foster City, CA, USA) were incubated with Opti-MEM (Invitrogen) and Lipofectamine™ RNAiMax reagent (Invitrogen) as previously described (24,25). We first confirmed the transfection efficiency of Pre-miR™ in cell lines, based on the down-regulation of *PTK9* mRNA by transfection with *miR-1* (as recommended by the manufacturer).

Cell proliferation assay. Cells were transfected with 10 nM siRNA or miRNA by reverse transfection and plated in 96-well plates at $3x10^3$ cells per well. After 72 or 96 h, cell proliferation was determined by the XTT assay, using the Cell Proliferation kit II (Roche Molecular Biochemicals, Mannheim, Germany) as previously described (24,25). Triplicate wells were measured for cell viability in each treatment group.

Cell apoptosis assay. Cells were transfected with 10 nM siRNA or miRNA by reverse transfection and plated in 6-well plates at $3x10^4$ cells per well. Cells were harvested 72 h after transfection by trypsinization and washed twice in cold PBS. Double staining with FITC-Annexin V and PI was carried out using the FITC Annexin V Apoptosis Detection Kit (BD Biosciences, Franklin Lakes, NJ, USA), according to the manufacturer's recommendations and analyzed within an hour



Figures 1. Regulation of *GSTP1* expression in HNSCC cell lines (HSC3 and SAS) by si-*GSTP1* transfection. (A) *GSTP1* mRNA expression was repressed after 72 h of transfection with 10 nM si-*GSTP1* compared with control transfectants. *P<0.05. (B) GSTP1 protein expression after 72 h transfection with si-*GSTP1*. β-actin was used as a loading control. The protein expression levels were also repressed in the transfectants.

by flow cytometry (FACScan®; BD Biosciences). Cells were resolved into viable cells, dead cells, early apoptotic cells and apoptotic cells by CellQuest software (BD Biosciences). The percentages of early apoptotic cells in each experiment were compared. Experiments were performed in triplicate.

Plasmid construction and the dual-luciferase reporter assay. miRNA target sequences were inserted between the *XhoI–PmeI* restriction sites in the 3'UTR of the *hRluc* gene in the psiCHECK™-2 vector (C8021; Promega, Madison, WI, USA). Primer sequences for the full-length 3'UTR of *GSTP1* mRNA (TAGGCGATCGCTCGAGGGGTTGGGGGGACTCTGA and TCTAGGTTTAAACTTTATTGGTCCTGGAGAAAGGA) were designed. SAS cells were transfected with 5 ng of vector, 10 nM miRNAs, and 1 μl of Lipofectamine™ 2000 (Invitrogen) in 100 μl of Opti-MEM™ (Invitrogen). The activities of firefly and *Renilla* luciferases in cell lysates were determined with a dual-luciferase assay system (E1910; Promega). Normalized data were calculated as the quotient of *Renilla*/firefly luciferase activities.

Results

Effect of GSTP1 silencing on cell proliferation in HNSCC cell lines. To examine the effect of silencing of GSTP1, si-*GSTP1* was transfected into HSC3 and SAS cell lines. The efficiency of both mRNA and protein silencing of *GSTP1* were evaluated in the siRNA transfectant cell lines. The expression levels of both mRNA and protein were clearly reduced in the si-*GSTP1* transfectant cells (Fig. 1).

We monitored cellular proliferation after si-*GSTP1* transfection in HSC3 and SAS cell lines. The XTT assay revealed that cell viability significantly decreased in a time-dependent manner in the si-*GSTP1* transfectants in comparison with the si-control in both HSC3 and SAS cells (Fig. 2). In HSC3 cells, the cell growth of si-*GSTP1* transfectants was 65.3% and 50.5% (both P<0.05) of that seen in si-controls after 72 h and 96 h, respectively. Similarly, in SAS cells, cell growth after 72 h and 96 h was 42.7%, and 35.2% of control, respectively (both P<0.05).

The effect of *GSTP1* silencing on apoptosis was analyzed using flow cytometry. In HSC3 cells, *GSTP1* silencing resulted in a 4.85-fold increase in the proportion of early apoptotic cells (Fig. 3, upper panels) while in SAS cells, it resulted in a 7.23-fold increase in the total proportion of early apoptotic cells after si-*GSTP1* transfection (Fig. 3, lower panels).

GSTP1 as a target of post-transcriptional repression by miR-133a. Interestingly, *GSTP1* 3'UTR contains a predicted target sequence for *miR-133a* suggesting it might function as a tumor suppressive miRNA. The expression levels of *GSTP1* mRNA and *GSTP1* protein were markedly reduced in *miR-133a* transfectant cells when compared to miRNA-control transfectants (Fig. 4). This suggests that *miR-133a* regulates *GSTP1* expression by cleavage or translational inhibition. We performed a luciferase reporter assay to determine whether *GSTP1* mRNA is an actual target site for *miR-133a*, as indicated by the TargetScan program (release 5.1, http://www.targetscan.org/). We constructed a vector encoding the full-length 3'UTR of *GSTP1* mRNA and found that the luminescence intensity

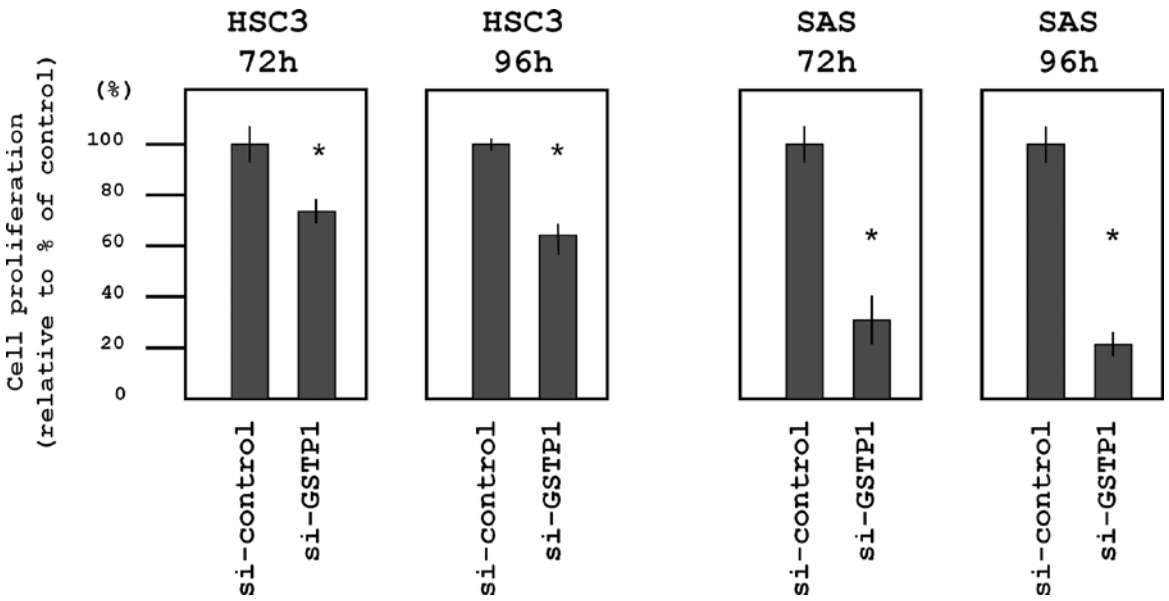


Figure 2. Effects of si-*GSTP1* transfection on the proliferation of HNSCC cell lines, HSC3 and SAS, by XTT assays. Inhibition of cell growth was observed in HSC3 and SAS cell lines 72 h or 96 h after transfection with 10 nM of si-*GSTP1* or control-siRNA. *P<0.05.

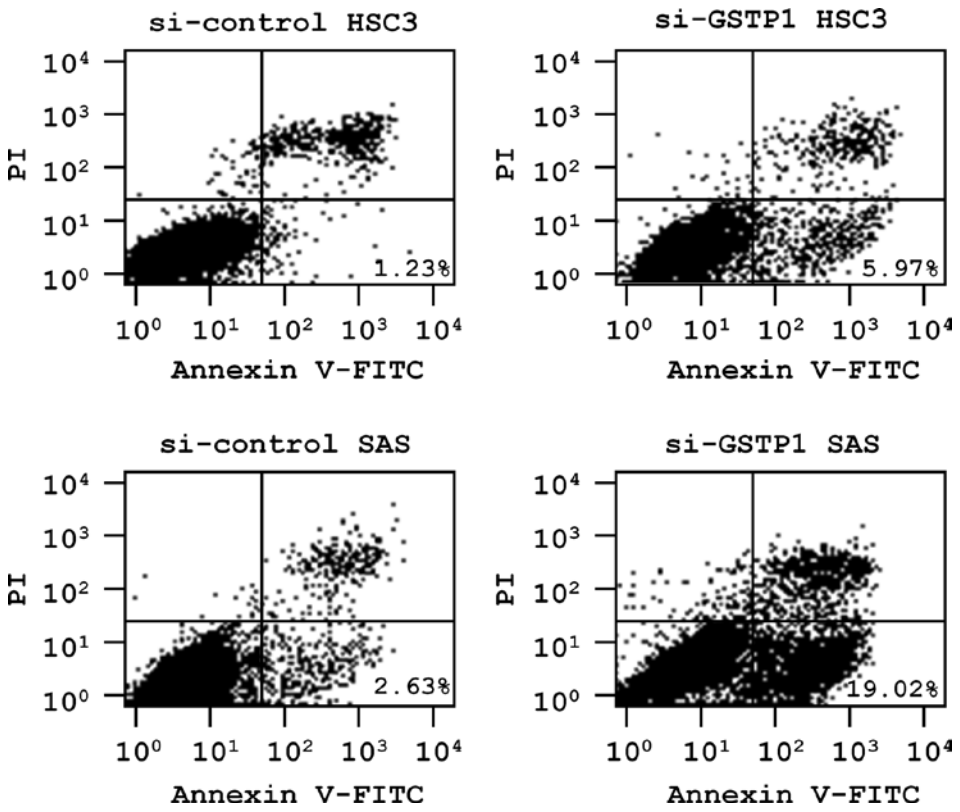
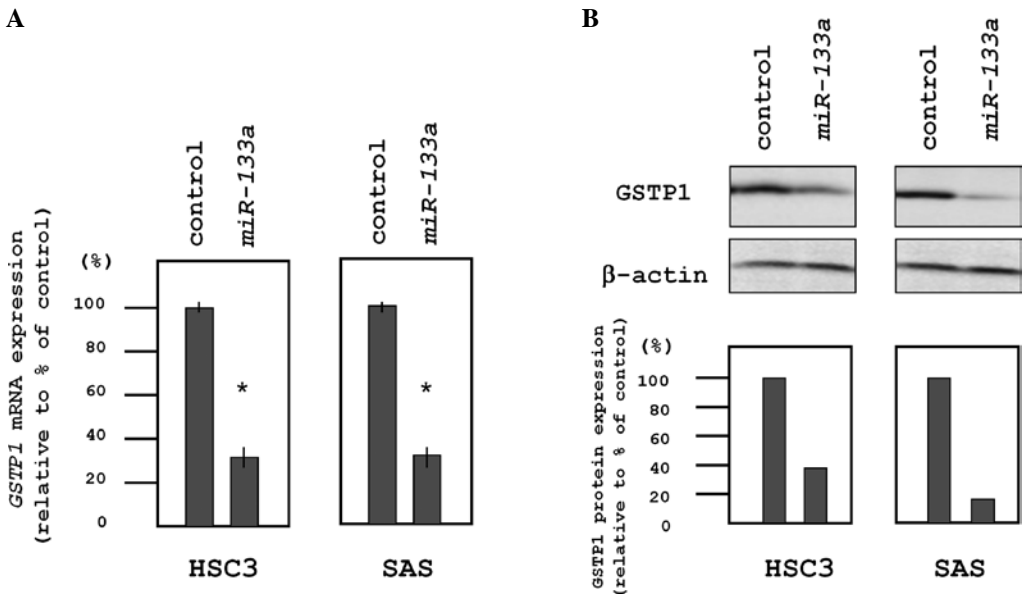


Figure 3. Flow cytometric analysis of si-*GSTP1* transfected HSC3 and SAS cells. Apoptotic cells increased 72 h after transfection of cells with 10 nM si-*GSTP1*.

was significantly lower (P<0.05) in the *miR-133a* transfectant (Fig. 5).

Underexpression of miR-133a and overexpression of GSTP1 protein in HNSCC clinical specimens. The expression levels of *miR-133a* were evaluated using 20 HNSCC clinical specimens. Levels of *miR-133a* were significantly lower (P=0.005) in cancerous tissues than in non-cancerous tissues (Fig. 6A). In contrast, the expression levels of *GSTP1* protein were up-regulated in cancer cells as determined by immunohistochemistry (Fig. 6B). Representative data of twenty HNSCC patients are described in Table I.



Figures 4. Regulation of *GSTP1* expression in HNSCC cells by *miR-133a* transfection. (A) *GSTP1* mRNA expression 72 h after transfection of *miR-133a*. *GSTP1* mRNA expression was reduced in both *miR-133a* transfectants. The results were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. *P<0.05. (B) GSTP1 protein expression was analyzed by Western blot analysis 72 h after transfection of *miR-133a*. GSTP1 protein expression was also reduced in *miR-133a* transfectants. The membranes were blotted with anti-GSTP1 IgG and anti-β-actin was used as a loading control. The density of each protein band was quantified using NIH-ImageJ. The results were standardized against the levels of β-actin.

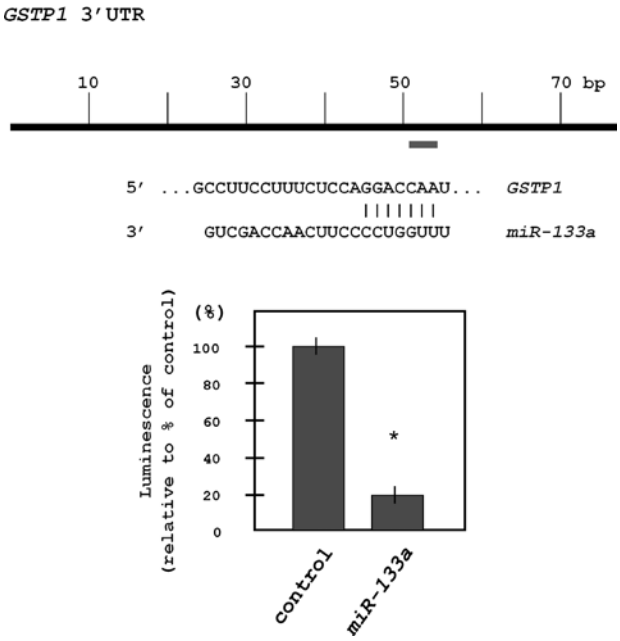


Figure 5. *miR-133a* regulates *GSTP1* expression in the SAS cell line at the mRNA and post-transcriptional level by targeting the 3'UTR of *GSTP1* mRNA. Schema for the putative conserved binding site of *miR-133a* (upper panel). The putative conserved target site in the *GSTP1* 3'UTR was identified with the TargetScan database. SAS cells were transfected with 5 ng of *GSTP1* 3'UTR in a vector construct or 10 nM of miRNA or negative control. *Renilla* luciferase activity was measured following a 24 h transfection. The results were normalized against firefly luciferase values (lower panel). *P<0.05.

Discussion

GSTs are phase II detoxification enzymes that catalyze the conjugation of electrophilic compounds with reduced glutathione (3). Among the GSTs, GSTP1 is frequently overexpressed in many cancers (5-10). Recently, we reported that mRNA levels of *GSTP1* were highly expressed in HNSCC tissues compared to adjacent non-cancerous tissues (4). Furthermore, *GSTP1* has been mapped to human chromosome 11q13 and this region is frequently altered in human HNSCC clinical specimens (26,27). Expression of *GSTP1* mRNA has been correlated with chromosomal alterations in HNSCC (4). However, the biological result of *GSTP1* overexpression in HNSCC remains unclear.

In this study, we explored silencing of *GSTP1* in two HNSCC cell lines, HSC3 and SAS. The analysis showed that *GSTP1* plays an oncogenic role by inhibiting apoptosis. Enhanced *GSTP1* expression might influence tumor growth by inhibition of apoptosis pathways, as reported in hepatic carcinoma, prostate cancer, small cell lung carcinoma, and myeloid leukemia (28-31). It has been shown that *GSTP1* protein interacts with c-Jun NH_2-terminal kinase (JNK) and tumor necrosis factor receptor-associated factor 2 (TRAF2), and suppresses the induction of apoptosis (32). In HEK293 cells, overexpression of *GSTP1* inhibited both MEKK1- and etoposide-induced apoptosis, and inhibited pro-caspase-3 activation and PARP cleavage (33). In clinical specimens of bladder cancer, BCL2 protein expression and high *GSTP1* expression were positively correlated (34). These findings are consistent with our report. The molecular interplay between *GSTP1* and the apoptotic cascade could provide new approaches for the development of cancer therapy.

Recently, it was reported that wild-type p53 regulates *GSTP1* expression and protects the genome from alkylating and free radical-generating compounds (35). Thus, it was of interest to determine which genes regulated *GSTP1*. We focused on miRNAs, an abundant class of small non-coding RNAs approximately 19-22 nucleotides in length that function as negative regulators of gene expression by antisense compli-

Table I. Clinical features of HNSCC patients.

No.	Gender	Age	Location	Differentiation	T	N	M
1	Male	60	Oral cavity	Well	2	0	0
2	Male	60	Oral cavity	Moderate	4	2c	0
3	Male	66	Oral cavity	Moderate	2	0	0
4	Female	73	Oral cavity	Well	1	0	0
5	Male	64	Oral cavity	Well	1	0	0
6	Male	66	Oral cavity	Well	3	0	0
7	Male	58	Oral cavity	Moderate	1	0	0
8	Male	73	Oral cavity	Poor	3	1	0
9	Female	38	Oral cavity	Well	2	2b	0
10	Male	66	Larynx	Well	4a	0	0
11	Male	69	Larynx	Well	3	0	0
12	Male	57	Larynx	Moderate	4a	0	0
13	Male	77	Oropharynx	Moderate	4b	2b	0
14	Male	52	Oropharynx	Moderate	3	2a	0
15	Female	65	Oropharynx	Well	4a	2b	0
16	Female	65	Hypopharynx	Well	4a	0	0
17	Male	68	Hypopharynx	Poor	4	1	0
18	Male	68	Hypopharynx	Moderate	4	1	0
19	Male	66	Hypopharynx	Moderate	2	2c	0
20	Female	74	Hypopharynx	Well	4a	0	0

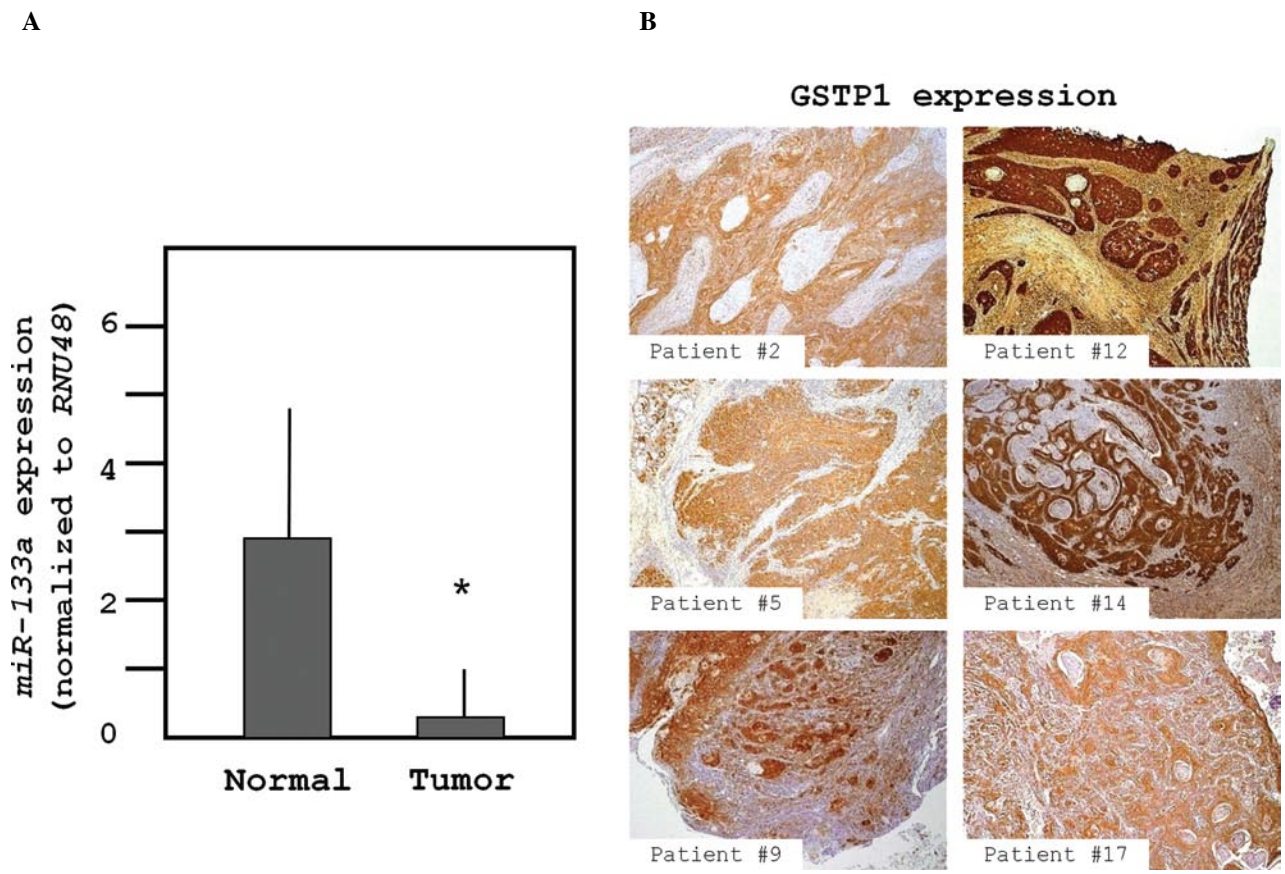


Figure 6. Expression levels of *miR-133a* and GSTP1 protein in HNSCC clinical specimens. (A) *miR-133a* expression in HNSCC specimens by real-time RT-PCR. *RNU48* was used as the internal control. *P=0.005. (B) GSTP1 protein expression in HNSCC specimens. Typical immunohistochemical results are shown (patients 2, 5, 9, 12, 14, and 17).

mentarity to specific messenger RNAs (13). A database search (TargetScan program: release 5.1, April 2009, http://www.targetscan.org/) revealed that *miR-133a* might target *GSTP1* mRNA. Our study proved that *GSTP1* expression was regulated through direct binding to a specific site in the 3'UTR.

Several studies indicated that *miR-133a* controls cardiac hypertrophy, and it is significantly down-regulated in hypertrophic and failing hearts (36). Targets of *miR-133a* are the Ras homolog gene family member A *(RHOA)* and the cell division cycle 42 *(CDC42)* genes (37,38). Expression of *miR-133a* is reduced in many cancers, including HNSCC and oral cancer, suggesting its role as a tumor suppressive miRNA (19-21,24,39). More recently, our studies demonstrated that *miR-133a* transfection of cancer cells significantly reduced cell proliferation, migration and invasion in bladder cancer, and esophageal squamous cell carcinoma. *miR-133a* directly regulated several oncogenic genes, such as *FSCN1* and *LASP1* (23,24,40). Other groups also found that *miR-133a* inhibited proliferation and induced apoptosis in squamous cell carcinoma of the tongue, and directly controlled pyruvate kinase type M2 gene expression (39). Thus, *miR-133a* regulates many oncogenic genes. How *GSTP1* overexpression changes in many types of cancers is still unclear, but one possible mechanism is through regulation by *miR-133a*.

Together, our findings demonstrate that the tumor suppressor *miR-133a,* regulates *GSTP1* expression and that overexpression of *GSTP1* increases cell proliferation by inhibiting apoptotic pathways in HNSCC. The therapeutic implications of these findings should be futher exploited towards the development of future HNSCC treatments.

Acknowledgements

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