Identification of Tumor-Suppressive miR-30e-3p Targets: Involvement of SERPINE1 in the Molecular Pathogenesis of Head and Neck Squamous Cell Carcinoma

Chikashi Minemura 1,2, Shunichi Asai 3,4, Ayaka Koma 1, Ikuko Kase-Kato 1, Nozomi Tanaka 1, Naoko Kikkawa 3,4, Atsushi Kasamatsu 1, Hidetaka Yokoe 2, Toyoyuki Hanazawa 1, Katsuhiro Uzawa 1 and Naohiko Seki 3,4*

Abstract: Recently, our studies revealed that some passenger strands of microRNAs (miRNAs) were closely involved in cancer pathogenesis. Analysis of miRNA expression signatures showed that the expression of miR-30e-3p (the passenger strand of pre-miR-30e) was significantly downregulated in cancer tissues. In this study, we focused on miR-30e-3p (the passenger strand of pre-miR-30e). We addressed target genes controlled by miR-30e-3p that were closely associated with the molecular pathogenesis of head and neck squamous cell carcinoma (HNSCC). Ectopic expression assays demonstrated that the expression of miR-30e-3p attenuated cancer cell malignant phenotypes (e.g., cell proliferation, migration, and invasive abilities). Our analysis of miR-30e-3p targets revealed that 11 genes (ADA, CPNE8, C14orf126, ERGIC2, HMGA2, PLS3, PSMD10, RALB, SERPINE1, SFXN1, and TMEM87B) were expressed at high levels in HNSCC patients. Moreover, they significantly predicted the short survival of HNSCC patients based on 5-year overall survival rates (p < 0.05) in The Cancer Genome Atlas (TCGA). Among these targets, SERPINE1 was found to be an independent prognostic factor for patient survival (multivariate Cox regression; hazard ratio = 1.6078, p < 0.05). Aberrant expression of SERPINE1 was observed in HNSCC clinical samples by immunohistochemical analysis. Functional assays by targeting SERPINE1 expression revealed that the malignant phenotypes (e.g., proliferation, migration, and invasion abilities) of HNSCC cells were suppressed by the silencing of SERPINE1 expression. Our miRNA-based approach will accelerate our understanding of the molecular pathogenesis of HNSCC.

Keywords: microRNA; HNSCC; miR-30e-3p; tumor suppressor; TCGA; SERPINE1

1. Introduction

The Global Cancer Statistics (2018) reported that head and neck squamous cell carcinoma (HNSCC) is the eighth most common human malignancy worldwide [1]. Every year, approximately 800,000 patients are diagnosed with HNSCC, and there are 430,000 deaths [2]. HNSCC is a malignant neoplasm that occurs in the oral cavity, hypopharynx, nasopharynx, and larynx, and the most common subtype is oral squamous cell carcinoma [3]. The aggressive nature of HNSCC is characterized by a high recurrence rate and distant metastasis. Almost 60% of such patients are initially diagnosed at an advanced stage of disease [4]. Treatment strategies for advanced cases are limited and their prognosis is poor, with 5-year...
survival rates below 30% [5]. The developed immunotherapies are not effective for many patients, and this treatment imposes a financial burden on patients [6]. In order to improve the prognosis of patients, it is essential to better understand the molecular mechanism underlying the malignant features of HNSCC and to develop new therapeutic strategies.

As a result of the human genome project, it became clear that a vast number of non-coding RNA molecules (ncRNAs) are transcribed from the human genome. Those molecules function in both normal and pathological cells [7]. Recent studies have revealed that various ncRNAs play pivotal roles in cell maintenance: for example, fine-tuning gene expression, controlling the cell cycle, and stabilizing RNA molecules, etc. [8,9]. It is apparent that the dysregulation of ncRNAs can contribute to the initiation and enhancement of human diseases, including cancers [10].

Due to their regulatory roles, microRNAs (miRNAs) have been intensively scrutinized. They function as fine-tuners of gene expression in a sequence-dependent manner [11]. A single miRNA might control a great number of RNA transcripts and contribute to various cellular signaling pathways under both normal and pathological conditions [10,12]. A vast number of studies have shown that aberrantly expressed miRNAs can function as oncogenes and/or tumor suppressors in human cancer cells, including HNSCC [13,14].

Two types of mature miRNAs are derived from pre-miRNAs. One strand (the guide strand) is selected for loading into the miRNA-Induced Silencing Complex (miRISC). The miRISC (including the guide strand) targets mRNA for silencing based on sequence compatibility [15]. The other strand of pre-miRNA (the passenger strand) is usually ejected and degraded in the cytoplasm [11,15]. However, recent studies showed that some passenger strands can act as oncogenes or tumor suppressors in a wide range of cancers, targeting several cancer-related genes [16–18]. Moreover, recent studies indicated that both strands of pre-miRNAs cooperated to control oncogenic pathways and exerted tumor-suppressive functions in several cancers [19–21]. The realization that passenger strands are involved in the molecular pathogenesis of cancer should permit new developments in cancer research.

We have created the miRNA expression signatures in various types of cancers [17,22,23]. Analysis of our miRNA signatures and other studies revealed that some members of the miR-30 family were frequently downregulated in cancer tissues, suggesting that the miR-30 family acted as pivotal tumor-suppressors [24–27]. The Cancer Genome Atlas (TCGA) datasets analysis showed that miR-30e-3p (the passenger strand derived from pre-miR-30e) was significantly downregulated in HNSCC tissues, and its low expression predicted worse prognosis of the disease. Some genes controlled by miR-30e-5p are closely associated with the prognosis of HNSCC patients [28,29]. In contrast, miR-30e-3p (the passenger strand) has not been carefully examined in HNSCC.

The aim of this study was to elucidate the involvement of miR-30e-3p in HNSCC. Here, ectopic expression assays showed that miR-30e-3p had tumor-suppressive roles in HNSCC cells. A total of 53 genes were successfully identified as putative miR-30e-3p targets in HNSCC cells. Among these targets, the high expression of 11 genes (ADA, CPNE8, C14orf126, ERGIC2, HMGA2, PLS3, PSMD10, RALB, SERPINE1, SFXN1, and TMEM87B) significantly predicted the short survival of HNSCC patients according to The Cancer Genome Atlas (TCGA) (5-year overall survival rate; \( p < 0.05 \)). This is the first report of the involvement of miR-30e-3p (the passenger strand) and its target genes in HNSCC.

2. Results
2.1. Expression Levels of miR-30e-3p in Clinical Specimens

The expression levels of miR-30e-3p were evaluated by TCGA-HNSC database analysis. The expression levels were significantly downregulated in HNSCC tissues compared with normal tissues (\( p < 0.001 \); Figure 1A). The downregulation of miR-30e-3p in HNSCC tissues was confirmed by other datasets (GSE45238 and GSE31277; Supplemental Figure S1). The expression of miR-30e-5p (the guide strand derived from pre-miR-30e) was also downregulated in HNSCC tissues by TCGA database analysis (data not shown). To determine the clinical significance, the 5-year overall survival rates of HNSCC patients were assessed.
using TCGA-HNSC data. Patients with low expression of miR-30e-3p had a significantly poorer prognosis compared to those with high expression [log rank p value = 0.0353, hazard ratio (HR) = 0.6097, 95% confidence interval (95% CI): 0.3828–0.9711] (Figure 1B).

![Figure 1A](image1.png)  
**Figure 1A.** miR-30e-3p expression levels in normal and HNSCC tissues. Expression levels of miR-30e-3p were significantly higher in normal tissues compared to HNSCC tissues. 

![Figure 1B](image2.png)  
**Figure 1B.** Kaplan–Meier survival analysis of HNSCC patients using TCGA database. Patients were divided into two groups (top 25% and low 25%). The red and blue lines indicate the high and low expression groups, respectively (log rank p value = 0.0353, HR = 0.6097, 95% CI: 0.3828–0.9711). 

![Figure 1C-E](image3.png)  
**Figure 1C-E.** Functional assays of cell proliferation, migration, and invasion following the transient transfection of miR-30e-3p in HNSCC cell lines (Sa3 and SAS cells). (C) Cell proliferation assessed by XTT assay at 72 h after siRNA transfection. (D) Cell migration assessed using a membrane culture system at 48 h after seeding miRNA-transfected cells into the chambers. (E) Cell invasion assessed by Matrigel invasion assays at 48 h after seeding miRNA-transfected cells into chambers.
2.2. Effects of Ectopic Expression of miR-30e-3p on HNSCC Cell Lines

To confirm the antitumor effectiveness of miR-30e-3p in HNSCC cells, we conducted ectopic expression assays using two HNSCC cell lines (Sa3 and SAS), focusing on cell proliferation, migration, and invasion. Cancer cell proliferation, migration, and invasion were significantly inhibited by miR-30e-3p transfection (Figure 1C–E). Representative images of the migration and invasion assays are shown in Supplemental Figure S2.

2.3. Identification of Putative Oncogenic Targets of miR-30e-3p in HNSCC Cells

To search for the oncogenic targets of HNSCC cells, we combined two datasets to select candidate targets controlled by miR-30e-3p. Our strategy for identifying miR-30e-3p target genes is shown in Figure 2.

According to the TargetScan database (release 7.2), 6118 genes had putative miR-30e-3p binding sites within their sequences (Figure 2). In this study, we performed genome-wide genes expression analysis (oligo-array) using miR-30e-3p transfected Sa3 cells, and 241 downregulated genes were identified. Our gene expression data were deposited in the Gene Expression Omnibus (GEO) database (accession number: GSE189290). We selected 150 genes by combining the aforementioned two datasets.

![Figure 2. Flowchart of the strategy used to identify candidate miR-30e-3p target genes in HNSCC cells.](image)

2.4. Clinical Significance of miR-30e-3p Target Genes Determined by TCGA Analysis

Clinicopathological analysis of the 150 putative target genes was performed using TCGA data to confirm clinical relevance. Among those 150 genes, 53 were significantly upregulated in HNSCC tissues (n = 518) compared with normal tissues (n = 44) according to the TCGA-HNSC database (Table 1).
Table 1. Upregulated genes in HNSCC clinical specimens in TCGA-HNSC database analysis.

| Entrez Gene ID | Gene Symbol | Gene Name                                                                 | Total Binding Sites | GEO log2 FC | 5y OS p-Value | FDR |
|---------------|-------------|---------------------------------------------------------------------------|---------------------|-------------|---------------|-----|
| 5054          | SERPINE1    | serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1 | 2                   | -1.98       | 0.0003        | 0.209 |
| 51290         | ERGIC2      | ERGIC and golgi 2                                                        | 2                   | -1.68       | 0.0032        | 0.0730 |
| 5358          | PLS3        | plasmin 3                                                               | 2                   | -1.53       | 0.0047        | 0.0898 |
| 94081         | SFXN1       | sideroflexin 1                                                          | 2                   | -1.92       | 0.0109        | 0.1423 |
| 8091          | HMGA2       | high mobility group AT-hook 2                                            | 1                   | -2.65       | 0.0131        | 0.1575 |
| 5716          | PSMD10      | membra (prosome, macropain) 26S subunit, non-ATPase, 10 transmembrane protein 87B | 1                   | -1.52       | 0.0204        | 0.2014 |
| 84910         | TMEM87B     | D-tyrosyl-tRNA deacetylase 2 (putative)                                  | 1                   | -1.64       | 0.0227        | 0.2137 |
| 112487        | C1orf126    | D-tyrosyl-tRNA deacetylase 2 (putative)                                  | 1                   | -1.58       | 0.0338        | 0.2663 |
| 100           | ADA         | adenosine deaminase                                                      | 1                   | -1.64       | 0.0355        | 0.2736 |
| 5899          | RALB        | v-ral simian leukemia viral oncogene homolog B                           | 2                   | -1.56       | 0.0370        | 0.2799 |
| 144402        | CPNE8       | copine VIII                                                             | 1                   | -1.74       | 0.0400        | 0.2921 |
| 10923         | SUB1        | SUB1 homolog (S. cerevisiae)                                             | 3                   | -2.53       | 0.0933        | 0.4584 |
| 6566          | SLC16A1     | solute carrier family 16 (monocarboxylate transporter), member 1         | 4                   | -1.75       | 0.0955        | 0.4640 |
| 3336          | HSPE1       | heat shock 10kDa protein 1 (chaperonin 10)                               | 1                   | -1.53       | 0.0960        | 0.4652 |
| 55156         | ARMC1       | armadillo repeat containing 1                                            | 4                   | -1.91       | 0.0997        | 0.4743 |
| 79624         | C6orf211    | chromosome 6 open reading frame 211                                      | 4                   | -1.50       | 0.1251        | 0.5316 |
| 528           | ATP6V1C1    | ATPase, H+ transporting, lysosomal 42kDa, V1 subunit C1                   | 2                   | -2.04       | 0.1309        | 0.5436 |
| 64841         | GNPNAT1     | glucosamine-phosphate N-acetyltransferase 1                             | 3                   | -1.73       | 0.1564        | 0.5927 |
| 10552         | ARPC1A      | actin related protein 2/3 complex, subunit 1A, 41 kDa                   | 1                   | -1.97       | 0.1871        | 0.6449 |
| 6780          | STAU1       | staufen double-stranded RNA binding protein 1                            | 3                   | -1.77       | 0.1883        | 0.6468 |
| 9265          | CYTH3       | cytohesin 3                                                              | 2                   | -1.52       | 0.2113        | 0.6817 |
| 90874         | ZNF697      | zinc finger protein 697                                                  | 3                   | -1.58       | 0.2182        | 0.6915 |
| 5923          | RASGRF1     | guanine nucleotide-releasing factor 1                                     | 2                   | -1.51       | 0.2259        | 0.7157 |
| 136           | ADORA2B     | adenosine A2b receptor                                                   | 1                   | -2.09       | 0.2367        | 0.7168 |
| 81539         | SLC38A1     | solute carrier family 38, member 1                                       | 5                   | -2.00       | 0.2493        | 0.7330 |
| 10473         | HMGN4       | high-mobility group nucleosomal binding domain 4                           | 2                   | -1.84       | 0.2678        | 0.7555 |
| Entrez Gene ID | Gene Symbol | Gene Name | Total Binding Sites | GEO log2 FC | 5-Year OS | p-Value | FDR |
|---------------|-------------|-----------|---------------------|-------------|-----------|---------|-----|
| 51762         | RAB8B       | RAB8B, member RAS oncogene family | 4           | −1.59       | 0.2683    | 0.7561  |
| 54165         | DCUN1D1     | DCN1, defective in cullin neddylation 1, domain containing 1 | 5           | −1.52       | 0.3327    | 0.8236  |
| 84056         | KATNAL1     | katanin p60 subunit A-like 1 | 2           | −1.55       | 0.3663    | 0.8530  |
| 3556          | IL1R1P      | interleukin 1 receptor accessory protein egl-9 family | 1           | −3.20       | 0.3916    | 0.8729  |
| 112399        | EGLN3       | hypoxia-inducible factor 3 | 1           | −2.10       | 0.4389    | 0.9051  |
| 1021          | CDK6        | cyclin-dependent kinase 6 complex 1 | 3           | −1.82       | 0.4733    | 0.9249  |
| 54108         | CHRAC1      | chromatin accessibility complex 1 thiopurine S-methyltransferase | 1           | −1.65       | 0.4733    | 0.9249  |
| 7172          | TPMT        | erythroblastosis virus E26 oncogene homolog 1 ninein (GSK3B interacting protein) | 3           | −1.89       | 0.5119    | 0.9436  |
| 51199         | NIN         | apelin | 2           | −1.58       | 0.5582    | 0.9616  |
| 8862          | APLN        | mitogen-activated protein kinase 6 | 1           | −2.05       | 0.5596    | 0.9621  |
| 5597          | MAPK6       | twisted gastrulation BMP signaling modulator 1 HAUS augmin-like complex, subunit 2 lysyl oxidase | 2           | −2.10       | 0.5818    | 0.9690  |
| 57045         | TWSG1       | nuclear autoantigenic sperm protein (histone-binding) phosphoprotein associated with glycosphingolipid microdomains 1 interleukin 1, beta transmembrane protein 237 | 1           | −1.69       | 0.5857    | 0.9701  |
| 55142         | HAUS2       | RAB23, member RAS oncogene family v-myb avian | 2           | −1.70       | 0.7820    | 0.9871  |
| 4678          | NASP        | myeloblastosis viral oncogene homolog-like 1 neuroblastoma RAS viral (v-ras) oncogene homolog family with sequence similarity 126, member A OTU domain containing 6B lamin B receptor ST3 beta-galactoside alpha-2,3-sialyltransferase 5 dihydrofolate reductase | 1           | −1.55       | 0.7897    | 0.9871  |
| 4893          | NRAS        | 3           | −2.53       | 0.7935    | 0.9871  |
| 84668         | FAM126A     | 2           | −1.61       | 0.8471    | 0.9871  |
| 51633         | OTUD6B      | 2           | −1.63       | 0.9276    | 0.9871  |
| 3930          | LBR         | 1           | −2.40       | 0.9492    | 0.9871  |
| 8869          | ST3GAL5     | 1           | −2.38       | 0.9686    | 0.9871  |
| 1719          | DHFR        | 4           | −1.96       | 0.9722    | 0.9871  |

1 Fold Change, 2 5-Year Overall Survival, 3 False Discovery Rate.

Furthermore, 11 of the genes (ADA, CPNE8, C14orf126, ERGIC2, HMGA2, PLS3, PSMD10, RALB, SERPINE1, SFXN1, and TMEM87B) predicted a significantly poorer
prognosis in HNSCC patients (Figures 3 and 4). To confirm the upregulation of these target genes in HNSCC tissues, we verified using other datasets (GSE30784 and GSE59102; Supplemental Figures S3 and S4). Furthermore, it was examined by quantitative PCR whether these target genes were regulated by miR-30e-3p in HNSCC cells. It was revealed that the expression levels of all genes were reduced by the miR-30e-3p transfected in HNSCC cells (Supplemental Figure S5). PCR primer sequences were shown in Supplemental Table S3. Among these targets, SERPINE1 was found to be an independent prognostic factor for patient survival (log rank test, \( p < 0.001 \) and false discovery rate < 0.05, multivariate Cox regression; hazard ratio = 1.6078, \( p = 0.0031 \); Table 1, Figure 5A).

**Figure 3.** HNSCC tissue expression of 11 target genes of miR-30e-3p using TCGA-HNSC data. The expression levels of 11 genes (ADA, CPNE8, C14orf126, ERGIC2, HMGA2, PLS3, PSMD10, RALB, SERPINE1, SFXN1, and TMEM87B) were analyzed using the TCGA-HNSC database. A total of 518 HNSCC tissues and 44 normal epithelial tissues were evaluated.
Figure 4. Five-year survival rates of 11 target genes of miR-30e-3p using TCGA-HNSC data. Kaplan–Meier survival analyses of HNSCC patients using data from the TCGA database. Patients were divided into high and low expression groups according to the median of each gene expression level. The red and blue lines indicate the high and low expression groups, respectively.

Moreover, we investigated the extent to which the expression of miR-30e-3p and SERPINE1 was correlated in HNSCC clinical specimens. Spearman’s rank test revealed a negative correlation between the expression levels of miR-30e-3p and SERPINE1 (p < 0.01, r = -0.3717; Figure 5B).

Immunohistochemistry was performed to analyze SERPINE1 protein expression in HNSCC clinical specimens. SERPINE1 protein was clearly stained in cancer lesions, whereas it was only weakly stained in normal tissues (Figure 5C).
Figure 5. Clinical significance of SERPINE1 using TCGA-HSCC data. (A) Forest plot showing the multivariate analysis results for SERPINE1, which were identified as independent prognostic factors for overall survival after adjustment for patient age, disease stage, and pathological grade. (B) Expression negative correlation between miR-30e-3p and SERPINE1 in HNSCC clinical specimens. Spearman’s rank test indicated negative correlations of miR-30e-3p expression with SERPINE1 (p < 0.01, r = −0.3717). (C) Immunohistochemical staining of SERPINE1 in HNSCC clinical specimens. SERPINE1 expression was high in the cancer lesions (right panels; b and d), whereas normal mucosa were only weakly stained (left panels; a and c).

2.5. Regulation of SERPINE1 Expression by miR-30e-3p in HNSCC Cells

Both the mRNA and protein expression levels of SERPINE1 were suppressed in miR-30e-3p-transfected HNSCC cells (Figure 6A,B). Full-size Western blot images are shown in Supplemental Figure S2. To investigate whether miR-30e-3p bound directly to the 3′-UTR of SERPINE1 in HNSCC cells, dual-luciferase reporter assays were performed. TargetScan database analysis revealed that there were two miR-30e-3p binding sites predicted within the
3′-UTR of SERPINE1 (Figure 6C). Luciferase activity was significantly reduced following the co-transfection of miR-30e-3p and a vector containing the miR-30e-3p-binding site (1322 to 1328) in the 3′-UTR of SERPINE1. On the other hand, co-transfection of a vector containing the SERPINE1 3′-UTR lacking the miR-30e-3p-binding site resulted in no change in luciferase activity (left panel of Figure 6C). With regard to the other predicted binding site (1607 to 1614), luciferase activity was not changed following co-transfection of miR-30e-3p and a vector containing the miR-30e-3p-binding site in the 3′-UTR of SERPINE1 (right panel of Figure 6C). Our present data suggest that miR-30e-3p binds directly to one of the predicted binding sites of SERPINE1, and that it controls the expression levels of SERPINE1 in HNSCC cells.

![Graphs and images](image-url)

**Figure 6.** Direct regulation of SERPINE1 expression by miR-30e-3p in HNSCC cells. (A) qRT-PCR showing significantly reduced expression of SERPINE1 mRNA at 72 h after miR-30e-3p transfection in Sa3 and SAS cells. Expression of GAPDH was used as an internal control. (B) Western blot showing reduced expression of SERPINE1 protein at 72 h after miR-30e-3p transfection in Sa3 and SAS cells. Expression of GAPDH was used as an internal control. (C) TargetScan database analysis predicting two putative miR-30e-3p-binding sites in the 3′-UTR of SERPINE1 (upper panel). Dual luciferase reporter assays showed reduced luminescence activity after co-transfection of the wild-type vector (position 1322–1328) and miR-30e-3p in Sa3 cells (left panel). There was no reduced luminescence activity after co-transfection of the deletion-type vector (position 1322–1328) and miR-30e-3p in Sa3 cells (lower panel). Normalized data are expressed as the Renilla/firefly luciferase activity ratio (N.S., not significant). For the other
putative binding site (position 1607–1614), there was no reduced luminescence activity after co-transfection of the wild-type vector and miR-30e-3p in Sa3 cells (right panel).

2.6. Effects of SERPINE1 Knockdown on the Proliferation, Migration, and Invasion Assays

To explore the potential cancer-promoting function of SERPINE1 in HNSCC cells, we used siRNAs targeting SERPINE1 in knockdown assays. First, the inhibitory effects of two different siRNAs targeting SERPINE1 (siSERPINE1–1 and siSERPINE1–2) on SERPINE1 expression were confirmed. The SERPINE1 mRNA and protein levels were effectively suppressed by each siRNA transfected into Sa3 and SAS cells (Supplemental Figure S3). Knockdown of SERPINE1 in HNSCC cells inhibited cancer cell malignant transformation (Figure 7A–C). Notably, cancer cell invasion and migration abilities were significantly blocked after siSERPINE1–1 or siSERPINE1–2 was transfected into Sa3 and SAS cells (Figure 7B,C). Our present data indicate that aberrantly expressed SERPINE1 contributed to the aggressive phenotype of HNSCC cells.

To understand the effects of overexpression of SERPINE1 in HNSCC cells, gene set enrichment analysis (GSEA) was performed to determine differentially expressed genes between the high and low SERPINE1 expression groups of the TCGA-HNSC cohort. The results of GSEA showed that the most enriched gene set in the high SERPINE1 expression group was “epithelial–mesenchymal transition” (Figure 7D and Table 2). GSEA analysis showed that several signal pathways were involved in the high SERPINE1 expression group, e.g., “myogenesis”, “TNFα signaling”, “angiogenesis”, and “KRAS signaling” (Table 2). We suggest that the overexpression of SERPINE1 may affect various intracellular signaling pathways. Activation of these signaling pathways may induce the malignant transformation of HNSCC cells.

Table 2. The significantly enriched gene sets in the high SERPINE1 expression group in TCGA-HNSC.

| Name                                      | Normalized Enrichment Score | FDR q-Value |
|-------------------------------------------|-----------------------------|-------------|
| HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION| 3.149                       | q < 0.001   |
| HALLMARK_MYOGENESIS                      | 2.866                       | q < 0.001   |
| HALLMARK_TNFA_SIGNALING_VIA_NFKB         | 2.489                       | q < 0.001   |
| HALLMARK_ANGIOGENESIS                    | 2.349                       | q < 0.001   |
| HALLMARK_KRAS_SIGNALING_UP               | 2.289                       | q < 0.001   |
| HALLMARK_COAGULATION                     | 2.285                       | q < 0.001   |
| HALLMARK_HYPOXIA                         | 2.282                       | q < 0.001   |
| HALLMARK_APICAL_JUNCTION                 | 2.247                       | q < 0.001   |
| HALLMARK_UV_RESPONSE_DN                  | 2.189                       | q < 0.001   |
| HALLMARK_INFLAMMATORY_RESPONSE           | 2.156                       | q < 0.001   |
| HALLMARK_INTERFERON_ALPHA_RESPONSE       | 2.054                       | q < 0.001   |
| HALLMARK_TGF_BETA_SIGNALING              | 2.027                       | q < 0.001   |
| HALLMARK_INTERFERON_GAMMA_RESPONSE       | 1.896                       | q < 0.001   |
| HALLMARK_COMPLEMENT                       | 1.846                       | q < 0.001   |
| HALLMARK_IL6_JAK_STAT3_SIGNALING         | 1.836                       | q < 0.001   |
| HALLMARK_NOTCH_SIGNALING                 | 1.793                       | 0.001       |
| HALLMARK_APOPTOSIS                       | 1.716                       | 0.002       |
| HALLMARK_HEDGEHOG_SIGNALING              | 1.692                       | 0.002       |
| HALLMARK_IL2_STAT5_SIGNALING             | 1.618                       | 0.004       |
| HALLMARK_GLYCOLYSIS                      | 1.593                       | 0.005       |
| HALLMARK_P53_PATHWAY                     | 1.396                       | 0.036       |
| HALLMARK_WNT_BETA_CATENIN_SIGNALING      | 1.376                       | 0.043       |
different siRNAs targeting SERPINE1 (siSERPINE1–1 and siSERPINE1–2) on SERPINE1 expression were confirmed. The SERPINE1 mRNA and protein levels were effectively suppressed by each siRNA transfected into Sa3 and SAS cells (Supplemental Figure S3). Knockdown of SERPINE1 in HNSCC cells inhibited cancer cell malignant transformation (Figure 7A–C). Notably, cancer cell invasion and migration abilities were significantly blocked after siSERPINE1–1 or siSERPINE1–2 was transfected into Sa3 and SAS cells (Figure 7B,C). Our present data indicate that aberrantly expressed SERPINE1 contributed to the aggressive phenotype of HNSCC cells.

Figure 7. Functional assays of cell proliferation, migration, and invasion following the transient transfection of siRNAs targeting SERPINE1 in HNSCC cell lines (Sa3 and SAS cells). (A–C) Functional assays of cell proliferation, migration, and invasion following the transient transfection of siSERPINE1–1 and siSERPINE1–2 in HNSCC cell lines (Sa3 and SAS cells). (A) Cell proliferation assessed by XTT assay at 72 h after siRNA transfection. (B) Cell migration assessed using a membrane culture system at 48 h after seeding miRNA-transfected cells into the chambers. (C) Cell invasion assessed by Matrigel invasion assays at 48 h after seeding miRNA-transfected cells into chambers. (D) GSEA analysis showed that most enrichment pathway was “epithelial–mesenchymal transition”.

3. Discussion

Initially, it was believed that only the guide strand derived from pre-miRNA was functional, whereas the passenger strand was degraded and had no function [11,15]. Contrary to this concept, a large number of studies have shown that the passenger strands of several miRNAs are responsible for the regulation of target genes in the cell [30]. A large number
of cohort analyses by TCGA data showed that in some miRNAs (e.g., miR-30a, miR-143, miR-145, and miR-139), both strands (-5p/-3p (the guide strand/the passenger strand)) cooperated to regulate oncogenic pathways [30]. Our recent studies demonstrated that miR-30a-3p, miR-143-5p, miR-145-3p, and miR-139-3p (all of which are passenger strands) acted as tumor-suppressive miRNAs through the control of many oncogenic genes and pathways in various types of cancers [31–33]. Finding functional passenger chains and exploring the networks they control will deepen our understanding of the molecular mechanisms of cancer.

The ectopic expression of miR-30e-5p markedly blocked the malignant characteristics of certain cancer cells, indicating that miR-30e-5p acted as a tumor-suppressor in HNSCC cells [28]. Our present analysis revealed that the other strand derived from pre-miR-30e also has a tumor-suppressive function. In other words, two miRNAs (miR-30e-5p and miR-30e-3p) derived from pre-miR-30e function as tumor suppressors in HNSCC cells.

Relatively little is known about the functional characteristics of miR-30e-3p in human cancers. A previous study showed that the overexpression of miR-30e-3p inhibited renal cell carcinoma (RCC) cell line (A498 and 786-O) migration and invasive abilities [34]. This study also indicated that Snail1 was directly regulated by miR-30e-3p in RCC cells [34]. Snail1 is a zinc finger-containing transcription factor. It functions as a negative regulator of E-cadherin expression, and it is closely related to RCC metastasis [35].

Recently, an interesting study of miR-30e-3p reported that miR-30e-3p possessed two functions (tumor-suppressor or oncogene) depending on TP53 status [36]. With wild-type TP53, miR-30e-3p targeted MDM2, and it seems to behave as a tumor suppressor. In contrast, with a nonfunctional TP53, miR-30e-3p behaved as an oncogene. Notably, elevated miR-30e-3p levels predicted the development of sorafenib resistance in hepatocellular carcinoma patients [36]. It is an interesting finding that the role of miR-30e-3p varies depending on the status of the TP53. It is necessary to examine whether this situation is a universal phenomenon in HNSCC cells.

A single miRNA can control a large number of genes, and the target genes can differ between cell types. Therefore, it is important to clarify which genes are controlled by miR-30e-3p for each type of cancer. Our in silico analysis of miR-30e-3p targets revealed that 53 genes acted as putative oncogenic targets in HNSCC. The expression of all 11 genes was closely associated with the molecular pathogenesis of HNSCC. A detailed analysis of these genes should improve our understanding of the molecular mechanisms underlying HNSCC. For example, Plastin-3 (PLS3) is an actin-bundling protein that contributes to cofillin-mediated actin polymerization [37]. The aberrant expression of PLS3 was reported in a wide range of cancers, and its expression is closely associated with the EMT-induced malignant phenotypes of cancers [38]. A previous study showed that the expression of PLS3 by circulating tumor cells was a marker in metastatic colorectal cancer [39]. Recent study indicated that it is possible to predict the responsiveness of lung cancer patients to nivolumab treatment by measuring plasma levels of PLS3 [40].

PSMD10 (Proteasome 26S Subunit, Non-ATPase 10), also known as Gankyrin, was initially cloned from a cDNA library obtained from a hepatocellular carcinoma. It consisted of ankyrin repeat motifs that promoted protein–protein interactions [41]. A large number of studies showed that aberrantly expressed PSMD10/Gankyrin was present in several types of cancers, and its overexpression enhanced cancer cell proliferation, invasion, and metastasis through the activation of several oncogenic signal pathways, e.g., RhoA/ROCK/PTEN, PI3K/AKT, and IL-6/STAT3 pathways [42]. In oral cancer, overexpressed PSMD10/Gankyrin was detected in cancer tissues and premalignant oral lesions [43].

Human RAL (RAS-like) is a member of the RAS-family (RAS, RAL, RIT, RAP, RHEB, and RAD), and it has an amino acid sequence most similar to RAS [44]. The human RALA and RALB genes were cloned from a human pheochromocytoma cDNA library. The two proteins share approximately 80% similarity at the amino acid level [45]. Previous studies showed that RALA and RALB might play distinctive oncogenic roles in human cancers,
as RALB contributed to the survival of human cancer cells [46]. In pancreatic cancer, RALB was activated in cancer tissues, and it enhanced the cells’ invasive ability and their metastatic colonization in vitro and in vivo studies [47].

SERPINE1 (also known as Plasminogen Activator Inhibitor-1: PAI-1) is an inhibitor of the plasminogen activators tPA and uPA [48]. In a wide range of cancers, the upregulation of SERPINE1 was observed in genome-wide gene expression analyses, and its expression is a marker of poor prognosis [49]. The roles of SERPINE1 in cancer progression have been studied in depth, especially tumor promotion of inflammation, sustaining proliferative signals, invasion and metastasis, angiogenesis, and resisting tumor death [50]. In genome-wide gene expression profiles of HNSCC, upregulated SERPINE1 was frequently observed in independent studies [51]. A previous study showed that the overexpression of SERPINE1 enhanced the migration of HNSCC cells, and its expression protected cells from cisplatin-induced apoptosis through activation of the PI3K/AKT pathway [52]. Another study indicated that overexpression of SERPINE1 also promoted tumor aggressiveness and metastatic dissemination to lymph nodes and lung. Moreover, its association is consistent with poor outcome in HNSCC patients [53]. Given its predominance in the HNSCC literature, it is surprising that no effective cancer chemotherapy targeting SERPINE1 has been proposed.

Accumulating studies have shown that miRNAs regulated the malignancy of cancer cells by controlling the target genes [54]. The downregulation of tumor-suppressive miRNAs causes the aberrant expression of oncogenic genes in cancer cells. The involvement of several tumor-suppressive microRNAs has been reported as a caused of overexpression of SERPINE1 in cancer cells [52]. Previous study showed that miR-617 was directly bound in the UTR of SERPINE1 mRNA and controlled its expression in OSCC cells [55]. Other study demonstrated that ectopic expression of miR-181c-5p suppressed the expression of SERPINE1 in HNSCC cells [56]. Notably, the expression of miR-617 and miR-181c-5p inhibited cancer cell proliferation and migration abilities in OSCC/HNSCC cells [55,56].

Here, we identified a number of genes that were controlled by miR-30e-3p. Many of those molecules are involved in cancer pathogenesis. Functional analysis of these genes will reveal at least some of the molecular mechanisms underlying HNSCC.

4. Materials and Methods

4.1. Transfection of Mature miRNAs and Small-Interfering RNAs (siRNAs) into HNSCC Cells

The HNSCC cell lines (SAS and Sa3) used in this study were obtained from the RIKEN BioResource Center (Tsukuba, Ibaraki, Japan). The miRNA precursors, negative control miRNA, and siRNAs were obtained from Applied Biosystems (Waltham, MA, USA). The procedures used for the transient transfection of miRNAs, siRNAs, and plasmid vectors were described in our previous studies [20,21,57,58]. miRNAs at 10 nM and siRNAs at 5 nM were transfected into HNSCC cell lines using RNAiMAX reagent (Invitrogen, Waltham, MA, USA). The reagents used in this study are listed in Supplemental Table S1; here, “mock”: transfection reagent only, and “control”: negative control miRNA precursor that have no function transfected.

4.2. Functional Assays of miR-30e-3p and SERPINE1 on HNSCC Cells

Cell proliferation, migration, and invasion assays were performed using HNSCC cells. The XTT assay (Sigma–Aldrich, St. Louis, MO, USA) characterized cell proliferation, a chamber assay using the Corning BioCoat™ cell culture chamber (Corning, Corning, NY, USA) assessed migration, and Matrigel chamber assays using the Corning BioCoat Matrigel assessed invasion. They were performed with HNSCC cells as described previously [20,21,57,58]. The reagents used in this study are listed in Supplemental Table S1.
4.3. RNA Extraction and Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

RNA extraction from cell lines and qRT-PCR were performed as described in our previous studies [20,21,57,58]. The TaqMan assays used in this study are summarized in Supplemental Table S1. The primers for SYBR green assays designed in this study are summarized in Supplemental Table S3. GAPDH was used as the internal control.

4.4. Western Blotting and Immunohistochemistry

Western blotting and immunohistochemical procedures were described in our previous studies [20,21,48,49]. Paraffin sections for immunohistochemistry were obtained from HNSCC patients who underwent surgical treatment at Chiba University hospital. The clinical features of the HNSCC patients are summarized in Supplemental Table S2. The antibodies used in this study are shown in Supplemental Table S1. Full blots of the membrane are shown in Supplemental Figure S2. Our study has been approved by the Ethics Committee of Chiba University (approval number; 28–65, 10 February 2015). The research methodology is implemented in accordance with the standards set by the Declaration of Helsinki.

4.5. Dual Luciferase Reporter Assays

Synthetic DNA sequences of SERPINE1 with or without the miR-30e-3p-binding sites were inserted into the psiCHECK-2 vector (C8021; Promega, Madison, WI, USA). The differences between wild-type and deletion type sequences are detailed in Figure 6. Then, the plasmid vectors and miR-30e-3p were transfected into Sa3 cells. After 48 h of transfection, dual luciferase reporter assays were performed using the Dual Luciferase Reporter Assay System (Promega). Normalized data are expressed as a Renilla/Firefly luciferase activity ratio.

4.6. Identification of miR-30e-3p Targets in HNSCC

To predict miRNA binding sites, we relied on the TargetScanHuman database (release 7.2, http://www.targetscan.org/vert_72/; accessed on 1 August 2019) [59]. We assessed the genes that were downregulated by the transfection of miR-30e-3p into HNSCC cells using genome-wide gene expression analysis (microarray assays). Our expression data were deposited in the GEO database (accession number: GSE189290). The strategy used for the identification of miR-30e-3p target genes in this study is summarized in Figure 2.

To validate the expression of miR-30e-3p and identified target genes in multiple HNSCC samples, GSE45238, GSE31277, GSE30784, and GSE59102 were downloaded from GEO datasets.

4.7. Analysis of miRNAs and miRNA Target Genes in HNSCC Patients

We used TCGA-HNSC (https://tcga-data.nci.nih.gov/tcga/; accessed on 10 July 2019) to investigate the clinical significance of miRNAs and their target genes. Clinical parameters and gene expression data were obtained from cBioPortal (http://www.cbioportal.org/; accessed on 13 July 2019) [60,61] and OncoLnc (http://www.oncolnc.org/; accessed on 1 August 2019) [62].

For Kaplan–Meier analyses of overall survival, we downloaded TCGA clinical data (Firehose Legacy) from cBioPortal (https://www.cbioportal.org; data downloaded on 13 July 2019). Gene expression grouping data for each gene were collected from OncoLnc (http://www.oncolnc.org/; accessed on 1 August 2019).

Monovariate and multivariate analyses were also performed using TCGA-HNSC clinical data and survival data according to the expression level of each gene from OncoLnc to identify factors associated with HNSCC patient survival. In addition to gene expression, the tumor stage, pathological grade, and age were evaluated as potential independent prognostic factors. The multivariate analyses were performed using JMP Pro 15.0.0 (SAS Institute Inc., Cary, NC, USA).
4.8. Gene Set Enrichment Analysis (GSEA)

To analyze the molecular pathways related to SERPINE1, we performed GSEA. We divided the TCGA-HNSC data into high and low expression groups according to the Z-score of the SERPINE1 expression level. A ranked list of genes was generated by log2 ratio comparing the expression levels of each gene between the two groups. The obtained gene lists were uploaded into GSEA software [63,64]. The Hallmark gene set in The Molecular Signatures Database was applied [63,65].

4.9. Statistical Analysis

All data are shown as mean values with standard errors derived from ≥3 independent experiments. Statistical analyses were determined using JMP Pro 15 (SAS Institute Inc., Cary, NC, USA). Welch’s t-test was performed to determine the significance of differences between two groups. Dunnet’s test was applied for comparisons among multiple groups. We applied Spearman’s rank test to evaluate the correlation between expression of miR-30e-3p and target genes. Monovariate and multivariate analyses were performed using Cox’s proportional hazards model. A p-value less than 0.05 was considered statistically significant.

5. Conclusions

In conclusion, analysis of the TCGA database showed that the expression level of miR-30e-3p was significantly downregulated in HNSCC clinical specimens. Ectopic expression assays demonstrated that miR-30e-3p inhibited HNSCC cells’ aggressiveness, suggesting that miR-30e-3p acted as a tumor suppressor. Interestingly, some of the putative targets regulated by miR-30e-3p closely predicted the prognosis of HNSCC patients. Our identification of miR-30e-3p (the passenger strand of pre-miR-30e) in the molecular pathogenesis of HNSCC may open the way to improved treatments of HNSCC oncogenesis.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Our expression data were deposited in the GEO database (accession number: GSE189290).

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