ANTICANCER RESEARCH *41*: 1785-1792 (2021)
doi:10.21873/anticanres.14944

Down-regulation of Glutathione Peroxidase 4 in Oral Cancer Inhibits Tumor Growth Through SREBP1 Signaling

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Abstract. *Background/Aim: This study aimed to elucidate the role of glutathione peroxidase 4 (GPX4) on the sterol regulatory element binding proteins (SREBPs)-proliferation pathway in oral cancer cells, and determine its protein expression in oral cancer tissues. Materials and Methods: Quantitative RT-PCR and immunoblot analysis were carried out. Cell viability assay, apoptosis detection assay, immunohistochemistry and GPX4 knockdown were performed. Results: The levels of both GPX4 mRNA and protein were highest in SAS cells. GPX4 knockdown in SAS cells, a human oral squamous cell carcinoma cell line, using GPX4 siRNA resulted in a reduction in cell number, which appeared to be due to non-apoptotic cell death such as ferroptosis. Furthermore, SREBP was clearly down-regulated by GPX4 knockdown in SAS cells. Immunopositivity for GPX4 was revealed on the membrane of human oral squamous cell carcinoma cells, and this was correlated with p53 immunoreactivity. Conclusion: GPX4 appears to play an important role in oral cancer proliferation.*

The most common epithelial oral cancer is human oral squamous cell carcinoma (HOSCC), which is characterized by a high infiltrative potential that can lead to early regional lymph node involvement and subsequent metastatic spread. Despite recent advances in multidisciplinary treatment modalities such as radiotherapy and/or chemotherapy, no improvement has been observed in the 5-year survival rate over the several decades (1). Moreover, considering traditional histopathological parameters, it remains difficult to predict the clinical course of HOSCC. For these reasons, gaining a better understanding of the molecular mechanisms underlying the local invasion of HOSCC has become a significant goal in the field of oral surgery. Local interactions between stromal and cancer cells have been described to play important roles in invasion and metastasis, such as in proteolytic degradation of the basal lamina, cellular detachment, migration across the extracellular matrix, and resistance to apoptotic cell death (2).

Glutathione peroxidases (GPXs), a type of selenoproteins, comprise a family of 8 members (GPX1 to GPX8), all of which have selenocysteine as the 21st amino acid residue (3). All eight members of the GPX family share the same basic function of peroxide reduction at the expense of glutathione. Despite the catalytic and structural similarities observed among the GPX isoforms, GPX4 is unique because it is the only enzyme that can reduce cholesterol hydroperoxides and esterified oxidized fatty acids (3, 4). For this reason, we focused on the functions of GPX4. Both the activity and expression of GPX4 depend on selenium (5). Among the different organs, the liver is particularly sensitive to selenium deficiency because other organs, including the brain and reproductive system, prioritize selenium uptake. Reactive oxygen species (ROS) associated with inflammation attack membrane lipids and form lipid hydroperoxides, promoting the propagation of oxidative hepatic damage (6), which GPX4 antagonizes by reducing the lipid hydroperoxides to the respective hydroxides. It has also been reported that GPX4 plays a substantial role in suppressing ferroptosis, a specific type of cell death (7). However, the role of GPX4 in human oral cancer is still unclear. Therefore, the aim of this study was to clarify the role of GPX4 in HOSCC.

On the other hand, the *p53* gene is a highly characterized tumor suppressor. This gene encodes a protein with a molecular mass of 53 kDa, which, in cells with sublethal genome damage at the late G_1 phase, has also been shown to act as a transcription factor capable of arresting the cell cycle until complete repair can be achieved or to induce apoptosis in cases of irreparable injury and activate further the

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Key Words: Glutathione peroxidase 4, ferroptosis, SREBP, p53, human oral squamous cell carcinoma.

transcription of specific genes (8, 9). Therefore, inactivation of the *p53* tumor suppressor gene through point mutation and allele loss is considered the most common genetic change underlying organ malignancies (10). In addition, these alterations appear to be related to the multi-step processes of oral carcinogenesis (11, 12). One study recently reported that, independent of cell-cycle arrest, senescence, and apoptosis, p53 induced ferroptotic cell death in the presence of ROS stress (13). In this study, we also examined whether GPX4 regulates the growth of oral cancer *in vitro*, and investigated the expression and distribution of GPX4 and p53 in HOSCC tissues, *in vivo*. Our findings suggested that GPX4 plays an important role in the proliferation and growth of oral cancer. Modification of GPX4 expression may be a novel approach for HOSCC treatment or prevention.

Materials and Methods

Reagents and chemical. For immunoblot analysis, rabbit anti-human GPX4 polyclonal antibody (PAb, GPX4) and sterol regulatory element binding protein 1 (SREBP1) PAb were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Novus Biologicals (Centennial, CO, USA), respectively. For immunohistochemistry, mouse anti-human p53 monoclonal antibody (Mab, p53) and GPX4 MAb were purchased from Cell Signaling Technology (CST; Tokyo, Japan) and Santa Cruz Biotechnology (Dallas, TX, USA), respectively. Ferrostatin-1 (Fer-1), identified as a small-molecule inhibitor of ferroptosis, was obtained from Sigma-Aldrich. A rabbit Mab against human β-actin was obtained from CST.

Cell culture. Various HOSCC cell lines [HSC-2, HSC-3, HSC-4, Ca9-22 and SAS; Japanese Cancer Research Resources Bank (JCRB), Osaka, Japan] were each cultured using 25-cm^2 culture flasks in RPMI-1640 medium, containing 10% heat-inactivated fetal bovine serum and 1% antibiotic-antimycotic (Life Technologies, Tokyo, Japan). Cells were grown to confluency at 37˚C in an atmosphere with 5% CO_2.

RNA extraction and real-time quantitative (q)RT-PCR. Total RNA was extracted from monolayered HOSCC cells (1×10^6 cells/ml) using a previously reported AGPC method (14). The expression patterns of the up-regulated or down-regulated *GPX4* and *SREBP1* genes were confirmed by qRT-PCR analyses using a Bio-Rad iCycler system (Nippon Bio-Rad Laboratories, Tokyo, Japan) and an iScript One-Step RT-PCR kit with SYBR Green I (Bio-Rad) in accordance with the manufacturer's instructions. The method has been previously described (15). The PCR primers were designed and synthesized by Sigma-Aldrich, Inc. (Ishikari, Japan). The following primer sequences were used in the PCR reactions: GPX4 forward: GAA GTA AAC TAC ACT CAG CTC; GPX4 reverse: CTC TTT GAT CTC TTC GTT ACT C; SREBP1 forward: AAT CTG GGT TTT GTG TCT TC; SREBP1 reverse: AAA AGT TGT GTA CCT TGT GG; GAPDH forward: CAG CCT CAA GAT CAT CAG CA. As an internal control, GAPDH mRNA was also used (set at 1); the primer sequences have been previously described (15).

Immunoblot analysis. Proteins were extracted from monolayered HOSCC cells (1×10^6 cells/ml) and the concentrations were determined

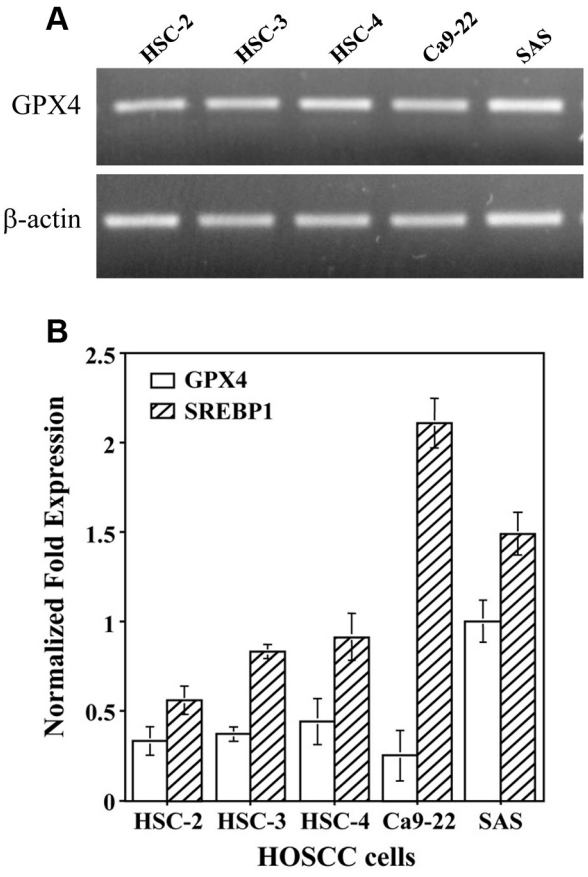

Figure 1. *Detection of GPX4 mRNA and the differences in the levels of GPX4 mRNA in human oral squamous cell carcinoma (HOSCC) cells. A) RT-PCR using total RNA from each HOSCC cell line was used to detect GPX4 mRNA. B) qRT-PCR showed that SAS cells had high levels of GPX4 and SREBP1 mRNA expression (n=3 experiments; means±SD).*

using a previously reported method (15). For detection of GPX4 and SREBP1 proteins, immunoblot analysis was performed as described method earlier (15). PAb GPX4 (1:1,000) or PAb SREBP1 (1:1,000) were used as primary antibodies. Then, horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (H+L) or anti-mouse IgG (H+L) antibody (1:25,000) (GE Healthcare, Piscataway, NJ, USA) were used as secondary antibodies. As an internal control, MAb β-actin (1: 5,000) was used.

RNA-mediated interference. Small interfering RNAs (siRNAs) specific for human GPX4 and scrambled (control) were synthesized by Sigma-Aldrich. The sense and antisense strand sequences of the oligonucleotides were as follows: GPX4 siRNA sense, GAG GCA AGA CCG AAG UAA AdTdT; antisense, UUU ACU UCG GUC UUG CCU CdTdT; MISSION® siRNA Universal Negative Control siRNA sense, AGU UUA UCG UAG GAA UUA U; antisense, AUA AUU CCU ACG AUA AAC U. FuGENE HD transfection reagent (Promega, Madison, WI, USA) was mixed with 10 nM GPX4 or 10 nM control siRNA (3:3.4 μl) in serum-free medium to a total volume of 500 μl, and then incubated for 30 min at room temperature. For GPX4 knockdown, SAS cells (derived from tongue

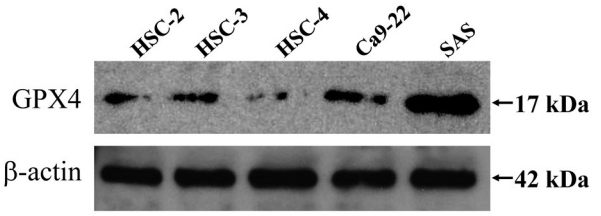

Figure 2. *Immunoblot analysis revealed that GPX4 is expressed in human oral squamous cell carcinoma (HOSCC) cells as a 17-kDa peptide and its expression levels were highest in SAS cells.*

cancer; 1×10^5 cells/ml) were rinsed with serum-free medium in 24-well plates and transfected with a GPX4 siRNA duplex or a control siRNA using FuGENE HD transfection reagents for 24 h at 37˚C. The cells were then subjected to cell viability assay, apoptosis detection assay and immunoblot analysis.

Cell viability assay. The method has been previously described (15). Briefly, SAS cells (2×10^4 cells/100 μl/well) were seeded and incubated in a 96-microwell plate, then treated with GPX4 siRNA or control siRNA for 48 h, followed by washing with PBS. WST-8/ECS solution (10 μl; Dojindo Laboratories, Tokyo, Japan) was then added to each well and incubation was continued at 37˚C in 5% CO_2 incubator for 4 h. The cells were well oscillated for 1 min, and absorbance was measured at 450 nm using a microplate reader. The SAS cells were then treated with GPX4 siRNA or control siRNA for 48 h with or without 10 μM Fer-1.

Apoptosis detection assay. Cells (5×10^3 cells/100 μl/well) were plated in white-walled 96-well cell culture plates and adhered for 24 h. After GPX4 knockdown for 24 h, Caspase-3/7, -8, and -9 activities were determined using the Caspase-Glo 3/7, -8, and -9 assay (Promega) as previously described (15).

Cell cycle analysis. The cell cycle analysis was performed as previously described (15) using a fluorescent ubiquitination-based cell cycle indicator (Fucci; Medical and Biological Laboratories (MBL), Nagoya, Japan). The original Fucci probe was generated by fusing monomeric Azami Green (mAG) and monomeric Kusabira Orange2 (mKO2) to ubiquitination domains of human Geminin and Cdt1, respectively. These two chimeric proteins, mAG-hGem and mKO2-hCdt1, accumulate reciprocally in the nucleus of transfected cells during the cell cycle, labeling the nuclei of $S/G_2/M$ phase cells green and those in G_1 phase red. mAG-hGem and mKO2-hCdt1 expression vectors were purchased from MBL. The SAS cells were transfected with mAG or mKO2 plasmid using Lipofectamine (Invitrogen, Carlsbad, CA, USA) for 24 h. Then, the transfected cells were subjected to knockdown with GPX4 siRNA or control siRNA, and observed by fluorescence microscopy.

Primary tumor samples. We obtained formalin-fixed, paraffin-embedded samples from 11 patients with oral SCC treated at our Hospital. The lesions were diagnosed pathologically by histological examination of hematoxylin and eosin (H-E) slides in accordance with the WHO classification (16). The postsurgical TNM stage was based on the International Union Against Cancer (UICC) pTNM

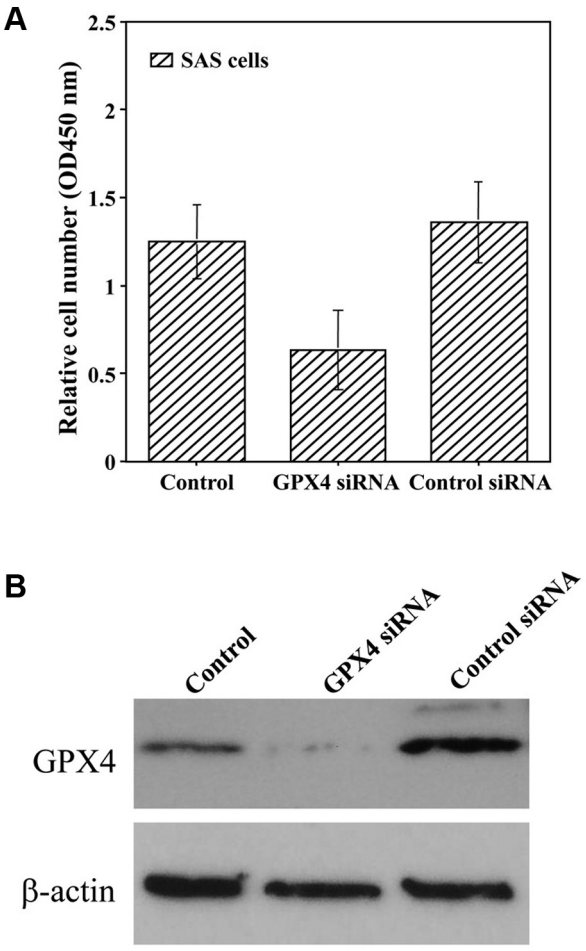

Figure 3. *Silencing GPX4 led to a reduction in the number of SAS cells. A) Cell viability analysis showing that GPX4 knockdown by GPX4 siRNA greatly reduced the number of SAS cells in comparison with the cells transfected with control siRNA or non-transfected cells (n=3 experiments; means±SD). B) Immunoblot analysis showing that GPX4 knockdown by GPX4 siRNA reduced GPX4 expression in SAS cells.*

pathological classification (17). All samples were obtained from surgical biopsies, and none of the patients had undergone radiotherapy or chemotherapy preoperatively.

Immunohistochemistry. For GPX4 and p53 immunostaining, the method has been previously described (15). Diluted MAb GPX4 (1:50) or MAb p53 (1:100) were used as primary antibodies. Then, horse anti-mouse IgG (H+L) antibody for GPX4 (1:200) or p53 (1:200) were respectively used as secondary antibodies.

Results

Expression of GPX4 gene in HOSCC cell lines. To investigate whether the cultured HOSCC cell lines (HSC-2, HSC-3, HSC-4, Ca9-22 and SAS) expressed GPX4 mRNA, we performed RT-PCR analysis using specifically designed

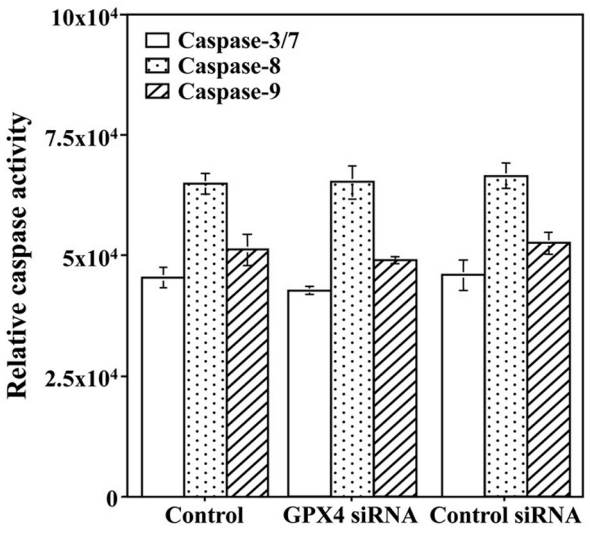

Figure 4. *Apoptosis detection assay showing that the levels of all caspases in SAS cells were not increased in response to GPX4 knockdown, following transfection with 10 nM GPX4 siRNA for 24 h (n=3 experiments; means±SD).*

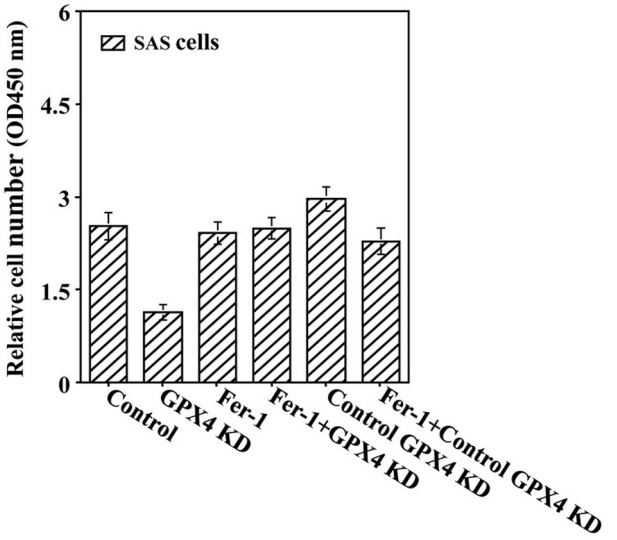

Figure 5. *Ferroptosis detection assay showing that although the number of SAS cells was decreased by GPX4 knockdown, this effect was inhibited by Fer-1. KD: Knockdown.*

Table I. *Correlation between the expression of p53, GPX4 and clinicopathological variables in 11 cases of oral squamous cell carcinomas.*

No.	Age	Gender	Location	Differentiation	pTNM	Stage	Immunohistochemistry	
							p53	GPX4
1	48	F	Gingiva	Well	T4N0M0	IVA	—	—
2	67	M	Mandibular gingiva	Well	T1N2bM0	IVA	+	+
3	66	F	Mandibular gingiva	Moderately	T1N0M0	I	—	—
4	65	M	Mandibular gingiva	Moderately	T2N0M0	II	—	—
5	88	M	Mandibular gingiva	Moderately	T2N0M0	II	—	+
6	64	M	Buccal mucosa	Poorly	T2N0M0	II	—	+
7	62	M	Tongue	Poorly	T2N1M0	III	+	+
8	60	M	Tongue	Poorly	T1N0M0	I	+	+
9	68	M	Maxillary gingiva	Poorly	T3N0M0	III	—	+
10	64	M	Maxillary gingiva	Well	T2N0M0	II	+	+
11	68	M	tongue	Well	T2N0M0	II	+	+

p53 expression: 5/11 cases (45.5%); GPX4 expression: 8/11 cases (72.7%).

primer pairs. Expression of GPX4 was detected in total RNA extracted from each cell line (Figure 1A). As an internal control, β-actin mRNA was detected to evaluate the integrity of the cDNA obtained from the total RNA extracted from the cell lines, and this confirmed that the total RNA was intact. To examine differences in the levels of GPX4 gene expression in HOSCC cells, qRT-PCR analysis was carried out. SAS cells showed the highest level of GPX4 mRNA expression and their levels of SREBP1 mRNA expression were also high (Figure 1B).

Expression of GPX4 protein in HOSCC cell lines. SDS-solubilized cell extracts of the HOSCC cell lines were subjected to immunoblot analysis to determine the expression of GPX4 protein and quantify their levels. This showed that HOSCC cell lines expressed GPX4 and its levels were highest in SAS cells (Figure 2).

GPX4 promotes proliferation of SAS cells. Using cell viability analysis, we next investigated whether GPX4 promotes the proliferation of SAS cells. An siRNA approach

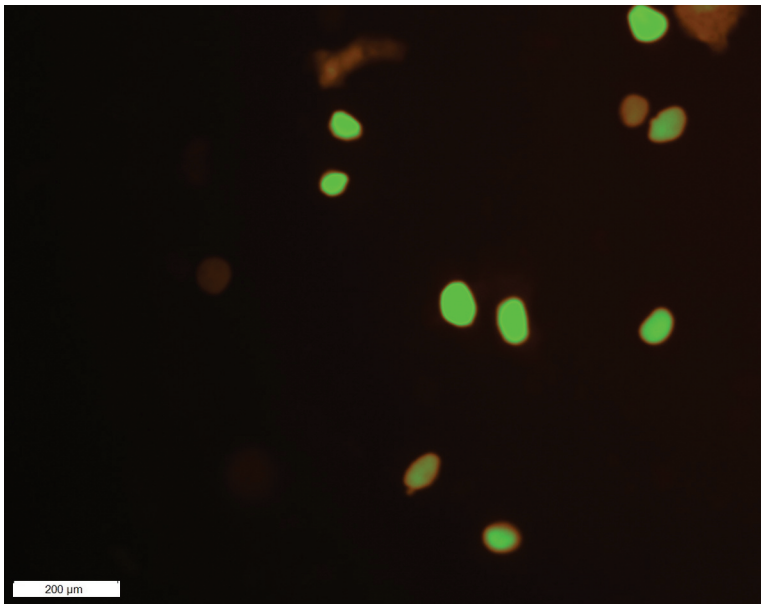

Figure 6. *Cell cycle analysis showing that GPX4 knockdown in SAS cells led to S/G_2/M phase arrest, but not G_1 phase arrest.*

was employed to reduce the expression of GPX4 and determine the number of SAS cells. As shown in Figure 3A, GPX4 knockdown by GPX4 siRNA obviously reduced the number of SAS cells in comparison with the cells transfected with control siRNA or non-transfected cells. It was then confirmed by immunoblot analysis that GPX4 knockdown by GPX4 siRNA indeed reduced the expression of GPX4 in SAS cells (Figure 3B). We considered that inhibition of SAS cell proliferation by GPX4 knockdown was a result of apoptosis, and therefore focused on SAS cells and performed an apoptosis detection assay.

GPX4 knockdown promotes cell death but not apoptosis in SAS cells. To determine whether caspase-dependent apoptotic activity could be induced by GPX4 knockdown, SAS cells were transfected with GPX4 siRNA or control siRNA for 24 h, and then the levels of procaspase cleavage yielding active caspase-8, -9, and -3/7 – markers of apoptotic activity – were examined. The levels of all caspases in SAS cells were not increased in response to GPX4 knockdown with 10 nM GPX4 siRNA for 24 h (Figure 4), suggesting that GPX4 knockdown led to non-apoptotic cell death. We then investigated whether this inhibition of cell proliferation by GPX4 siRNA was due to ferroptosis. For this purpose, SAS cells were treated with 10 μM Fer-1 before GPX4 knockdown. SAS cell viability was reduced by the treatment with GPX4 siRNA, but pretreatment with Fer-1 inhibited this GPX4 siRNA-induced cell death (Figure 5), suggesting cell death through ferroptosis. We then used Fucci to analyze the

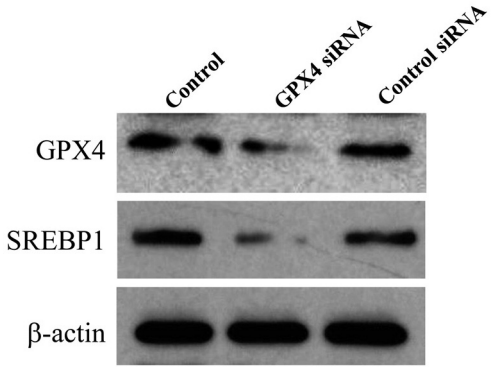

Figure 7. *GPX4 knockdown led to a reduction in SREBP1 expression. The levels of GPX4 protein in SAS cells were markedly reduced by treatment with GPX4 siRNA in comparison with the cells transfected with control siRNA or non-transfected cells. This GPX4 knockdown led to a marked decrease in SREBP1 activation in comparison with the cells transfected with control siRNA or non-transfected cells.*

phase at which SAS cell cycle was arrested after knockdown by GPX4 siRNA. This revealed that SAS cells subjected to GPX4 knockdown were arrested in S/G_2/M phase, but not in G_1 phase (Figure 6).

GPX4 regulates SREBP1 activation in SAS cells. To investigate how SREBP1, a nuclear transcriptional factor related to lipid metabolism, was regulated in SAS cells upon GPX4 knockdown, we used siRNA to reduce the expression

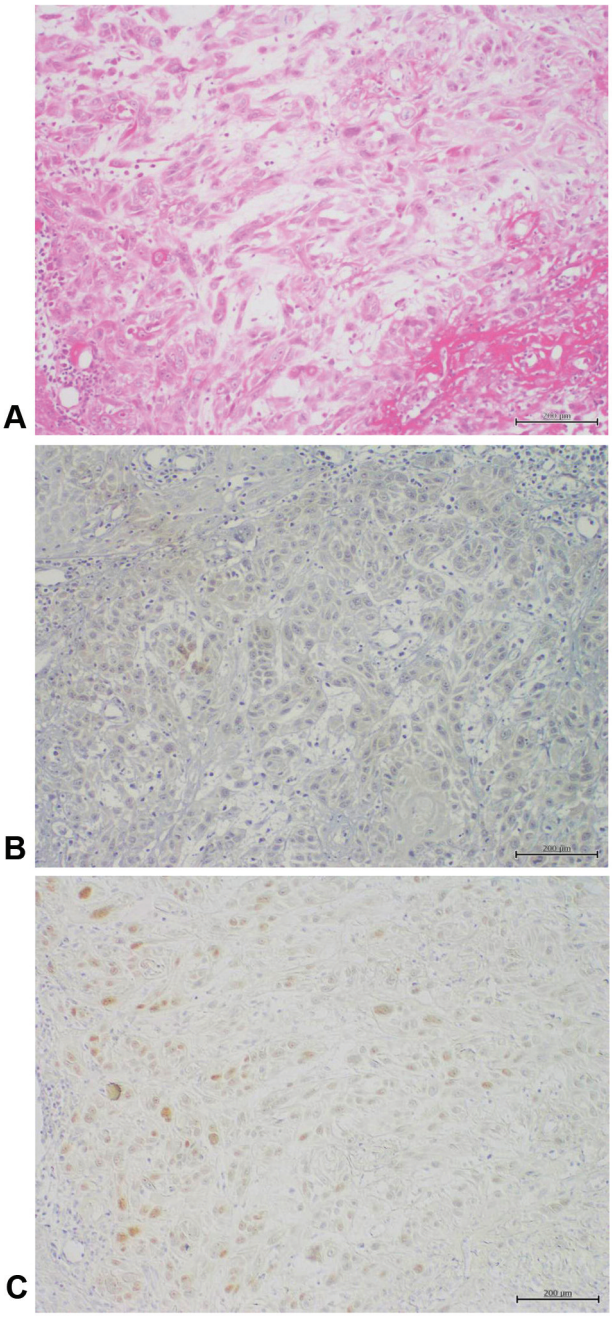

Figure 8. *Immunohistochemical detection of GPX4 and p53 in human oral squamous cell carcinoma (HOSCC) tissues. A) The invasive front of HOSCC was observed by H-E staining. B) Positive immunoreactivity for GPX4 was observed in the tumor cell cytoplasm in 8 of 11 (72.7%) HOSCC tissue samples. C) Meanwhile, p53 immunoreactivity was clearly observed in the tumor cell nuclei in 5 (45.5%) of the HOSCC tissue samples, all of which were GPX4-positive.*

of GPX4 and examine the resulting effects on the basal activity of SREBP1, as evaluated by immunoblot analysis. The levels of GPX4 protein were notably reduced by GPX4 siRNA in SAS cells relative to the cells transfected with control siRNA or non-transfected cells (Figure 7). We then evaluated the effect of GPX4 siRNA on SREBP1-dependent transcriptional activity. As shown in Figure 7, GPX4 knockdown led to a marked decrease of SREBP1 activation in SAS cells in comparison with the cells transfected with control siRNA or non-transfected cells. These findings indicated that endogenous GPX4 functions as a regulator of SREBP1 activation in SAS cells, further suggesting that ferroptotic cell death was induced in SAS cells by GPX4 knockdown.

Immunohistochemical detection of GPX4 and p53 in HOSCC tissues. The correlations between GPX4, p53 expression, and clinicopathological variables in HOSCC tissues are summarized in Table I. Immunohistochemical detection of GPX4 and p53 was performed in 11 cases of HOSCC. The invasive front of HOSCC was observed by H-E staining (Figure 8A). Positive reaction for GPX4 was observed in the tumor cell cytoplasm in 8 (72.7%) of the samples (Figure 8B). Meanwhile, positive reaction for p53 was clearly observed in the tumor cell nuclei in 5 (45.5%) of the samples (Figure 8C), all of which were GPX4-positive.

Discussion

Oral cancer accounts for approximately 3-5% of all cancer cases (18, 19). Unlike cancers in other parts of the body, damage to the facial region can cause substantial psychological distress and negatively affect patients' daily lives, particularly speaking and eating. Even though clinical outcomes of HOSCC have improved, it is still accompanied by a risk of invasion to adjacent organs and an increased possibility of metastatic recurrence. A combination of surgical therapy, radiation, and medication is typically used to treat oral cancers; however, severe adverse events, poor treatment efficacy, and the ineffective inhibition of cancer cell growth and micrometastases can lead to a poor prognosis. Substantial interest has therefore been expressed in exploring improved oral cancer treatments, and new diagnostic and prognostic biomarkers are considered necessary to optimize clinical management and therapeutic decision-making.

GPX4 is among the most important antioxidant and lipid repair enzymes in mammals, and a growing number of studies has recognized the key role it plays as a negative regulator of ferroptosis, a non-apoptotic form of cell death characterized by the production of iron-dependent ROS, through limiting lipid hydroperoxide levels (20–23). The oncogenic RAS-selective lethal small molecule erastin has recently been shown to induce ferroptosis by inhibiting cysteine uptake by the cysteine/glutamate antiporter, resulting in the iron-dependent accumulation of lethal lipid ROS and even ferroptotic cell death, which is biochemically, genetically, and morphologically distinct from both necrosis

and apoptosis. A number of studies have provided evidence of ferroptosis in the cells of a variety of malignant tumors, including fibrosarcoma (24), lung cancer (25), osteosarcoma (26), and prostate cancer (27). However, it is still unclear whether ferroptosis occurs in oral cancer. Furthermore, the role of GPX4 in oral cancer is also unknown.

In this study, therefore, we evaluated the cell growth of GPX4-positive oral cancer. As real-time qRT-PCR and immunoblot analysis indicated over-expression of GPX4 mRNA and protein in SAS cells, these cells were investigated further. To clarify how GPX4 functions in SAS cells, GPX4 knockdown analysis was carried out, and this revealed that the number of SAS cells was reduced as a result. Then, to confirm whether this reduction in SAS cell number was caused by apoptosis, caspase activities were analyzed, and the results suggested that GPX4 knockdown did not cause apoptotic cell death. Although the number of SAS cells was decreased as a result of GPX4 knockdown, this effect was inhibited by Fer-1, suggesting that the underlying process was, in fact, ferroptotic cell death. Furthermore, it was found that the cell cycle of SAS cells was arrested at $S/G_2/M$ phase during this cell death process. Thus, it was demonstrated that expression of GPX4 increased the proliferation of oral cancer cells *in vitro*, whereas GPX4 knockdown by siRNA inhibited tumor growth. These results are consistent with recent reports on the function of GPX4 in hepatocellular carcinoma (6) and breast cancer (28).

On the other hand, although SREBPs, a family of transcription factors, are known to regulate lipid biosynthesis and adipogenesis by controlling the expression of several enzymes required for fatty acid, cholesterol, phospholipid, and triacylglycerol synthesis (29), it is still unclear whether SREBPs correlate with GPX4 expression in oral cancer. We showed here that SREBP1 activation was clearly down-regulated by GPX4 knockdown in SAS cells. These data imply that GPX4 expression increased SREBP1 activation in SAS cells, whereas GPX4 knockdown led to ferroptosis. Taken together, these data suggest that GPX4 produced by oral cancer cells promotes tumor growth.

Both the initiation and progression of HOSCC involve a series of genetic events, including the inactivation of tumor suppressors and the activation of oncogenes (30). In addition, p53 has been shown to be a central regulator of cell growth, proliferation, and apoptosis (31) and ferroptosis (32) in genetic studies. Our *in vivo* studies demonstrated GPX4-specific immunoreactivity in HOSCC biopsy samples, which was correlated with p53 positivity in the cells. As the half-life of wild-type p53 is only 6-30 min, typically, the protein cannot be detected by immunohistochemical examinations; however, in the case of DNA damage, it accumulates and becomes detectable (33). Our finding suggests that p53 generally functions as a negative regulator of GPX4, however, the damaged p53 becomes inactive, and then, it is increased GPX4 expression. In addition, the SAS cells used in this study showed the point mutation of p53 gene. It can therefore be hypothesized, based on both the present *in vitro* and *in vivo* observations, that a loss of p53 function results in SREBP1 and GPX4 activation, thereby inhibiting ferroptosis, and consequently, may promote HOSCC tumorigenesis. Then, to clarify further the nature of GPX4-mediated tumor proliferation, and to establish a GPX4-based therapeutic strategy for oral cancer, further investigations into the role of GPX4 are needed. Nonetheless, these findings suggest that the modification of GPX4 expression could be a novel approach for the treatment and/or prevention of HOSCC.

Conflicts of Interest

The Authors declare no conflicts of interest with regard to the present study.

Authors' Contributions

Masakatsu Fukuda performed and analyzed all *in vitro* and some *in vivo* experiments, and wrote the manuscript; Yudai Ogasawara carried out some of the *in vivo* studies; Hideaki Sakashita supervised and coordinated this study, as well as provided research resources. All Authors read, reviewed, and approved the manuscript.

Acknowledgements

This work was supported by a Grant-in-Aid for scientific research from the Ministry of Education, Science, and Culture of Japan.

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Received January 13, 2021
Revised February 19, 2021
Accepted February 22, 2021