Running Title: S100A4 modulates head and neck cancer initiating cells

The Epithelial-Mesenchymal Transition Mediator S100A4 Maintains Cancer Initiating Cells in Head and Neck Cancers

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Abstract

Cancer initiating cells (CICs) are a rare subpopulation of cells in tumors that are proposed to be responsible for tumor growth. Starting from CICs identified in head and neck squamous cell carcinomas (HNSCCs), termed head and neck cancer initiating cells (HN-CICs), we determined as a candidate stemness-maintaining molecule for HN-CICs the pro-inflammatory mediator S100A4, which is also known to be an inducer of epithelial-mesenchymal transition (EMT). S100A4 knockdown in HN-CICs reduced their self-renewal capability and their stemness and tumorigenic properties, both in vitro and in vivo. Conversely, S100A4 overexpression in HNSCC cells enhanced their stem cell properties. Mechanistic investigations indicated that attenuation of endogenous S100A4 levels in HNSCC cells caused downregulation of Notch2 and PI3K/pAKT along with up-regulation of PTEN, consistent with biological findings. Immunohistochemical analysis of HNSCC clinical
specimens showed that S100A4 expression was positively correlated with clinical grading, stemness markers and poorer patient survival. Together, our findings reveal a crucial role for S100A4 signaling pathways in maintaining the stemness properties and tumorigenicity of HN-CICs. Further, our findings suggest that targeting S100A4 signaling may offer a new targeted strategy for HNSCC treatment by eliminating HN-CICs.
Introduction

Accumulating data support the hierarchical model of cancer initiating cells (CICs) or cancer stem cells (CSCs) in that each tumor formation is governed by a rare subpopulation of cells with self-renewal capacity (1, 2). CICs have been demonstrated to have capacities of promoting tumor growth, tumor regeneration, metastatic progression and contributing to radio-resistance and chemo-resistance (3, 4). We previously enriched a subpopulation of head and neck cancer initiating cells (HN-CICs) from HNSCC cells (HNSCCs) by sphere formation assay (5). The enriched HN-CICs possess both the properties of stemness and malignant tumors. However, it is still elusive with regards to the molecular mechanistic understanding leading to the HN-CICs phenotypic properties.

Epithelial-mesenchymal transition (EMT), a process by which epithelial cells lose their polarity and later acquire a migratory mesenchymal phenotype, is one of the crucial processes
that induce tumor invasion and metastasis (6). Researchers have shown that EMT could promote stem cells (SCs) properties and further generate cells with the features of breast CSCs (7-9). Therefore, how modulators of EMT process operating or manifesting the stem-like properties and the tumorigenicity of HN-CICs is warranted to shed light for future research.

S100A4, a member of calcium-binding proteins (CBPs), is directly controlled by Wnt/\(\beta\)-catenin signaling pathway as a master mediator in EMT (10). Involved in a variety of biological effects including cell motility, survival, differentiation and cytoskeletal organization (11-14), S100A4 was also demonstrated to play an important role in both stem cell and cancer biology. For instance, S100A4 is considered to be a normal stemness marker and plays a crucial role in the self-renewal of bulge stem cells (11, 13, 14). Mice lacking S100A4 gene suppresses the tumor development and metastasis (15). S100A4 is also
established as a regulator of metastasis while S100A4 is ectopically overexpressing in tumor cells, consequently, promotes metastatic phenotype (16, 17). In contrast, inhibition of S100A4 expression reduces the metastatic capacity of tumor cells (18). Recent data point out that S100A4 is highly expressed in human embryonal carcinoma cells (ECC) but not in human embryonic stem cells (ESC) by a comprehensive quantitative proteomic analysis (19).

In addition, S100A4 is significantly up regulated in mouse glioma CSCs (20). Others have shown the prognostic significance of S100A4 in many solid tumors including breast cancer, colon cancer, and bladder cancer (21-23). However, the role of S100A4 in HNSCC has not been well characterized.

Herein, we demonstrate that alteration of S100A4 expression affects CICs properties in HNSCCs. Additionally; immunoactivity of S100A4 on HNSCC tumor tissues correlates with clinical grading, survivals and stemness markers. Thus, our study implicated that S100A4
played an important role in the pathogenesis of HNSCC, and S100A4 might be a potential therapeutic target for HNSCC.

Materials and Methods

Cell lines cultivation and enrichment of HN-CICs from HNSCCs

Two well established HNSCC cell lines (SAS and OECM1) and one primary HNSCC cell line, used in this research, were derived from head and neck squamous cell carcinomas (5). In brief, originally, SAS and primary HNSCC were grown in DMEM, and OECM1 was grown in RPMI supplemented with 10% fetal bovine serum (FBS) (Grand Island, NY), respectively. For enrichment of HN-CICs, the above three cell lines were then cultured in tumor sphere medium consisting of serum-free DMEM/F12 medium (GIBCO), N2 supplement (GIBCO), 10 ng/mL human recombinant basic fibroblast growth factor-basic (bFGF) and 10 ng/mL
Epidermal Growth Factor (EGF) (R&D Systems, Minneapolis, MN) (24).

Microarray differential expression analysis

Gene profiling was performed using Affymetrix Human Genome U133plus2.0. All CEL files were pre-processed using ‘justRMA’ and standardized with mean of zero and SD of 1.

The fuzzy c-mean (FCM) algorithms of ‘Mfuzz’ package was used to analyze temporal gene expression patterns of our SAS HN-CICs (25). We focused the analysis on 63 EMT-related genes (204 probesets). Parameters in FCM were set as suggested (m=1.25; c=6) (25).

Functional annotation of gene clusters was done by the web-based program of DAVID (Database for Annotation Visualization and Integrated Discovery) (26). Modified t-test of ‘limma’ package (27) was used for differential gene expression analysis between the control- or S100A4-knockdown HN-CICs, controlled for FDR<0.05 (28). Two manually curated
gene sets were used: 1999 EMT and calcium signaling-related genes (4235 probesets) (EMT-Calcium)(29) and, 3939 stemness genes (8606 probesets) (ESC)(30, 31).

Network Analysis of Human Protein-Protein Interactions

Perturbed genes after shRNAi-mediated knockdown of S100A4 were mapped in the protein-protein interactions (PPIs) downloaded from the Human Protein Reference Database (32). Interactions would be mapped only when both of the interacting genes were listed in the EMT-Calcium or ESC sets. Topological characteristics were examined among the 1st and 2nd-order connecting neighbors of the mapped genes, that is, subnetworks of the shortest path of a maximum of 3 between any pair of these significantly perturbed genes (29). Analytical analyses were performed in R environment (33), and displayed by Cytoscape (34).
Aldehyde dehydrogenase activity analysis

Aldehyde dehydrogenase activity was examined by using ALDEFLUOR kit (Stem cell Technologies, Durham, NC, USA), and was performed according to manufacturer’s guidelines (35).

Side population analysis.

Cells were resuspended at 1×10^6/ml in pre-warmed DMEM with 2% FCS. Hoechst 33342 dye was added at a final concentration of 5μg/ml in the presence or absence of verapmil (50 μM; Sigma) and was incubated at 37°C for 90 min. The cells were then washed with ice-cold HBSS with 2% FCS. Propidium iodide at a final concentration of 2μg/ml was added to the cells to gate viable cells. The Hoechst 33342 dye was excited at 357 nm and its fluorescence was dual-wavelength analyzed (blue, 402–446 nm; red, 650–670 nm). Analyses were done on
a FACS Vantage (BD, San Diego, CA).

**Subcutaneous xenografts in nude mice**

All the animal practices in this study were in accordance with the institutional animal welfare guideline of Taipei VGH, Taiwan. Cells were injected subcutaneously into BALB/c nude mice (6-8 weeks). Tumor volume (TV) was calculated using the following formula: TV (mm³) = (Length × Width²) / 2 and then analyzed using Image Pro-plus software.

**Patient Subjects and Immunohistochemistry.**

Between 1994 and 1997, 102 patients with operable head and neck cancer, without histories of radiation or chemotherapy, underwent surgery at the Department of Oral and Maxillofacial...
Surgery, Mackay Memorial Hospital. This research follows the tenets of the Declaration of Helsinki and all samples were obtained after informed consent from the patients. Patients’ tissue samples with different stages of oral cancer were spotted on glass slides for immunohistochemical staining (Supplementary Table 1). After deparaffinization and rehydration, antigen retrieval was processed within 1X-Trilogy buffer (Biogenics). The slides were immersed in 3% H₂O₂ for 10 minutes and washed then blocked with serum (Vestastain Elite ABC kit, Vector Laboratories, Burlingame, CA), and followed by incubating with the primary anti-S100A4 antibody (Code No. A5114, from Dako, Glostrup, Denmark (36-38)). Tissue slides were then stained with biotin-labeled secondary antibody and incubated with streptavidin-horse radish peroxidase conjugates. Afterwards, the tissue sections were immersed with chromogen 3-3’-diaminobenzidine plus H₂O₂ substrate solution (Vector® DBA/Ni substrate kit, SK-4100, Vector Laboratories, Burlingame, CA). Hematoxylin was
applied for counter-staining (Sigma Chemical Co., USA). Pathologists scoring the immunohistochemistry were blinded to the clinical data. The interpretation was done in five high-power views for each slide, and 100 cells per view were counted for analysis.

**Statistical analysis.**

Statistical Package of Social Sciences software (version 13.0) (SPSS, Inc., Chicago, IL) was used for statistical analysis. The independent Student’s *t*-test or ANOVA was used to compare the continuous variables between groups, whereas the $\chi^2$ test was applied for the comparison of dichotomous variable. The Kaplan-Meier estimate was used for survival analysis, and the log-rank test was selected to compare the cumulative survival durations in different patient groups. The level of statistical significance was set at 0.05 for all tests.
Results

Elevated expression of S100A4 in head and neck cancer initiating cells (HN-CICs).

Cells undergoing EMT processes promote the gain of stem-like properties in breast carcinoma cells (8, 9). Therefore, we were interested in knowing if EMT-related genes were differentially expressed in the enriched HN-CICs from SAS cells under 2, 3, 5 and 9 weeks of cultivation within defined serum-free selection medium. We observed a clear separation of EMT-related gene expression patterns in 6 clusters without redundancy (Figs. 1A, 1B and Supplementary Figs. S1A and S2A). Cluster 4 demonstrated a significant increasing trend of gene activities (Figs. 1A). S100A4, a well-known player in the EMT and metastasis processes, was identified in cluster 4 showing induced activities in HN-CICs (Figs. 1A and 1B).

Functional annotation of cluster 4 showed enrichment in EMT, mesenchymal cell differentiation, and cell development (Fig. 1C). Empirically, the amount of S100A4
transcripts of enriched HN-CICs derived from both SAS and OECM1 HNSCCs were significantly increased in comparison with that of the parental HNSCCs, by either real-time PCR (Fig. 1D, *left panel*) or reverse transcriptase-PCR (RT-PCR) analysis (Supplementary Fig. S1B). Accordingly, the western blotting data showed that the protein levels of S100A4 in enriched HN-CICs were also up-regulated (Fig. 1D, *middle panel*). Furthermore, immunofluorescent staining displayed that the intracellular levels of S100A4 in the tumor spheres derived from SAS cells were dramatically increased (Fig. 1D, *right panel*).

**Effect of S100A4 knockdown on HNSCC and HN-CICs.**

To further investigate the crucial role of S100A4 up-regulation in maintaining the biological properties of HN-CICs, we performed loss-of-function approach by small hairpin RNA interference (shRNAi)-mediated knockdown of S100A4 in HNSCCs. Stable
knockdown of S100A4 in SAS, primary HNSCC and OECM1 cells was achieved by transduction with lentivirus expressing shRNA targeting S100A4 (Sh-S100A4-1 and Sh-S100A4-2), and lentivirus expressing shRNA against luciferase was used as control. The amount of S100A4 transcript was significantly decreased in S100A4 stable knockdown HNSCCs by real-time PCR analyses (Supplementary Fig. S3A left panels). Western blot analysis further confirmed that both sh-S100A4-1 and sh-S100A4-2 markedly reduced S100A4 protein expression in both HNSCCs (SAS and primary HNSCC cells) (Fig. 2A, left panels).

As successful sphere formation of CSCs under serial passages is a key behavior of normal SCs and CSCs for evaluating in vitro self-renewal property (39), we then determined the sphere formation capacity of HNSCCs with stable knockdown of S100A4. Knockdown of S100A4 markedly decreased the ability of HNSCCs to form tumor spheres (Supplementary
Fig. S3A, *right panels*), as well as indicated by the reduction in sphere formation efficiency after serial passages (Fig. 2A, *right panels*). In addition, the enzymatic activity of aldehyde dehydrogenase (ALDH), which has been identified as a CSC marker (35), was also significantly reduced in HNSCCs with S100A4 down-regulation (Fig. 2B, *left panels*). It has been demonstrated that tumor-derived side population (SP) cells display the characteristics of cancer stem cells (40). Here we showed that S100A4 depletion significantly decreased the side population in primary HNSCC and OECM1 cells (Fig. 2B, *middle and right panels*, and Supplementary Fig. S3B, *left panels*). Further, stable knockdown of S100A4 also decreased ABCG2 positive cells where high expression of ABCG2 possibly contributes to SP phenotype and drug-resistance in many cancers (Supplementary Fig. S3B, *right panels*) (41).

In addition, stable S100A4-knockdown HNSCCs also dramatically decreased “cancer stemness” genes (Oct-4 and Nanog) expression (Supplementary Fig. S3C).
To further investigate whether S100A4 expression plays a role in maintaining self-renewal or cancer stem-like properties of HN-CICs directly, the SAS or primary HNSCC–derived tumor spheres, afterward, transduced with Sh-S100A4 lentivirus did not maintain floating spheres but showed more attached epithelial-like cells (Fig. 2C). Instead, tumor spheres after Sh-S100A4 lentiviruses infection displayed enhanced expression of epithelial differentiation marker, CK18 (Fig. 2D, left and middle panels) and also decreased “cancer stemness” genes (Oct-4 and Nanog) expression (Supplementary Fig. S3D). To determine whether the reduction in tumor sphere formation efficiency with S100A4 down-regulation is due to decreased HN-CICs survival, we determined the percentage of apoptotic cells using Annexin V staining. Primary HNSCC or SAS-derived HN-CICs transduced with Sh-S100A4 lentivirus significantly increased the percentage of Annexin V–positive cells (Fig. 2D, right panel and data not shown). Together, these data further
support that the depletion of S100A4 resulted in a reduction of CICs population in HNSCCs.

The cell migratory/invasion/colony formation abilities of HNSCCs (SAS and OECM1) with S100A4 stable knockdown were also significantly reduced (Supplementary Figs. S4A, S4B and S4C). Furthermore, S100A4 stable knockdown abrogated EMT signatures with up-regulation of E-cadherin and down-regulation of vimentin by immunoblotting analyses (Supplementary Fig. S4D).

**Down-regulation of S100A4 attenuates tumorigenicity of HNSCCs *in vivo***.

We next sought to determine if down-regulation of S100A4 expression reduces the tumor forming ability of HNSCCs *in vivo*. As shown in figure 3A, SAS control cells generated tumor when $2.5 \times 10^5$ cells were injected into nude mice (six out of six mice), however, stable S100A4-knockdown SAS cells inefficiently gave rise to a new tumor at
5x10^5 in one of six mice. In addition, knockdown of S100A4 in SAS cells significantly reduced the tumor volumes (Fig. 3B; upper panel and middle panel, * p<0.05) and prolonged the survival of nude mice (Fig. 3B; lower panel, ** p<0.01). Our data indicate that down-regulation of S100A4 diminished the tumorigenicity of HNSCCs. Next, we addressed if targeting S100A4 could represent a potential therapeutic treatment. We first injected parental SAS cells into nude mice, and allowed the tumors to be established for 12 days. Tumor-bearing mice were then injected intratumorally with lentivirus expressing either Sh-Luc as a control or Sh-S100A4 as a therapeutic treatment. Apparently, tumor-bearing mice receiving lentivirus expressing Sh-S100A4 displayed retarded tumor growth (Fig. 3C; upper panel and middle panel, * p<0.05) and prolonging lifespan (Fig. 3C; lower panel, ** p<0.01).
Overexpression of S100A4 in HNSSCs enhances stemness properties and tumorigenic potentials

To evaluate whether overexpression of S100A4 could enhance the stemness and tumorigenic properties of HNSCCs, we generated stable S100A4-overexpressing HNSCCs through lentiviral-mediated transduction. Total proteins from S100A4-overexpressing HNSCCs displayed elevated expression of S100A4 and vimentin, but decreased expression of E-cadherin (Fig. 4A; left panel). The S100A4-overexpressing HNSCCs also showed significantly enhanced tumor sphere-forming capacity; both in size and number, within 2 weeks of cultivation under defined serum-free medium (Figs. 4B and Supplementary Fig. S5A). Moreover, S100A4-overexpressing HNSCCs, under cultivation with defined serum-free medium for 2 weeks, displayed increased protein level of Oct-4 and Nanog (Fig. 4C). The S100A4-overexpressing HNSCCs also displayed significantly increased SP cells.
Furthermore, we demonstrated that S100A4 overexpression also resulted in increased ability on \textit{in vitro} cell migration (Supplementary Fig. S5C) and cell invasion (Supplementary Fig. S5D). Collectively, these results suggest that S100A4 overexpression promotes stemness properties and \textit{in vitro} tumorigenicity of HNSCCs.

\textbf{S100A4 immunohistochemistry study in HNSCC patients.}

The expression profile of S100A4 in oral squamous cell carcinoma (OSCC) has been evaluated with controversial results (42, 43). The mRNA level of S100A4 is significantly down-regulated in 27 cases of OSCCs/their pair-wised normal controls obtained from Sudanese patients (43). However, Moriyama-Kita et al demonstrate that positive correlation of S100A4 expression with invasion and metastasis in 41 primary OSCC tissues of Japan
patients (42). The controversy could be from different patient populations or sample sizes.

To thoroughly investigate the expression profile of S100A4 during the development of head and neck cancers in HNSCC patients we established the ontogeny of S100A4 expression by tissue immunohistochemical staining with a panel of specimens array of 102 HNSCC patients. The clinicopathological features of the subjects are summarized in Table 1. We observed that elevated expression of S100A4 was highly correlated with medium to poor differentiation (p<0.0001), tumor stage (p<0.0001), lymph node metastasis (p<0.0001) and advanced staging (p<0.0001) of head and neck cancers (Figs. 5A and 5B). Additionally, we found more nuclear and cytoplasmic staining of S100A4 in the moderate to poor-differentiated HNSCC tissues than those of well-differentiated HNSCC tissues (Fig. 5A).
Poor overall survival rate and high recurrence of HNSCC patients was positively associated with S100A4 expression.

In order to determine the prognostic significance of S100A4 expression in patients with HNSCC, Kaplan-Meier survival analysis was performed. The Kaplan-Meier survival analyses showed that an overall worse survival rate was associated with the S100A4 IHC-positive patients in comparison with the negative ones (Fig. 5C; left panel). In addition, HNSCC patients with intense S100A4 expression were also associated with greater recurrence status (Fig. 5C; right panel). Together, these results demonstrate a significantly positive correlation between higher expression of S100A4 and tumor progression in HNSCC.

**Coexpression profile between S100A4 and stemness markers, Nanog, Oct-4 and CD133, of HNSCC.**
Further, we wanted to understand the expression relationship between S100A4 and the known stemness markers (Nanog, Oct-4 and CD133) from our previous findings on HNSCC (5). Of the 34 HNSCC patients’ tumorous tissues, which were previously immunohistochemically stained with Nanog, Oct-4 or CD133, respectively (5), we observed the significant co-expression between S100A4 and Nanog (p<0.001) (Fig. 5D; left panel), and S100A4 and Oct-4 (p<0.05) (Fig. 5D; middle panel) but not in S100A4 and CD133 (p=0.138) (Fig. 5D; right panel) with further staining against S100A4 antibodies.

S100A4 knockdown causes significant changes of calcium signaling, EMT, ESC, developmental (Notch2) and cell survival (PTEN/Akt/PI3K) related transcriptomes.

By examining transcriptomic changes after shRNAi-mediated knockdown of S100A4 in HNSCCs, 35 genes in EMT-Calcium (Supplementary Fig. S6A) as well as 78 genes in ESC
genesets (Supplementary Fig. S6B) were perturbed. Inter-relationships among the perturbed
genes were mapped in the human PPIs (Fig. 6A). S100A4, with 14 neighbors in the
EMT-Calcium networks, was the only connecting hub for MYH4, SEPT, and PPFIBP1 (Fig.
6A, inset). ACTA1, TPM3, and TP53 were also highly connected (Fig. 6A, inset). Network
topological analysis among the 1st and 2nd-order neighbors of the mapped perturbed genes
highlighted important hubs in the major subnetwork demonstrating that EMT-Calcium
processes might be ‘inter-regulated’ with the stemness behaviors. First, a significant overlap
among the EMT-Calcium and ESC genesets was noticed (Fig. 6A and Supplementary Figs.
S6A and S6B). Second, some of the perturbed genes such as CD47, NOTCH2, TPM3 and
NFYB resided as significant hubs linking the EMT-Calcium and ESC interactions. However,
despite those genes such as ACTA1, CAV, CASP3, ESRI, EGFR, SP1, and TP53 were
likewise connecting other inter-modular hubs, we did not find significant changes of gene

27
activity. To further study the possible mechanisms involved in S100A4-mediated stemness and tumorigenic properties, we showed that knockdown of S100A4 decreased Notch2, p-Ak/PI3Kt, and increased PTEN expression in HNSCCs (Fig. 6B and Supplementary Figs. S6A and S6B). These results suggested that Notch and PTEN-PI3K-Akt signaling played a significant role in mediating CICs characteristics; and moreover, S100A4 might inter-regulate to modulate such HN-CICs behaviors.

Discussion

In the present study, we directly evaluated the role of S100A4 in the maintenance of stemness characteristics and tumorigenic potential of HNSCCs by lentiviral shRNAi-mediated knockdown and lentiviral-mediated overexpression of S100A4 (Figs. 2, 3 and 4). Depletion of S100A4 decreased the stemness properties of HNSCCs and HN-CICs
both *in vitro* and *in vivo* (Figs. 2 and 3). In contrast to S100A4 knockdown experiments, overexpression of S100A4 enhances tumor sphere-forming capability, side population cells and promotes migration/invasion ability of HNSCCs (Fig. 4). Furthermore, analysis of the cell survival and differentiation ability of isolated HN-CICs revealed that loss of S100A4 caused a reduction of the CICs subpopulation and an increasing of the apoptotic and differentiated cells in HN-CICs (Fig. 2). Knockdown of S100A4 also lessened tumor initiating activity (Figs. 3). These results indicate that S100A4 directly contributes to the self-renewal and survival of HN-CICs cells. In addition, our clinical data indicate that higher S100A4 expression correlates with HNSCC tumor progression and lymph node metastasis, as well as contributes to patient mortality and relapse (Fig. 6). Of note, the expression profile of S100A4 is significantly correlated with stemness marker such as Nanog and Oct-4 but not CD133 (Fig. 5D) in HNSCC. All above suggest that stemness properties
mediated by S100A4 indeed play instrumental roles in the tumorigenicity of HNSCC. At last, through transcriptome profiling analysis again we discovered that knockdown of S100A4 affected EMT-Calcium-ESC related genes, such as TP53, NOTCH2, PTEN, and PI3K (Fig. 6).

The Notch and PTEN-PI3K-Akt signaling pathways have been demonstrating for regulating self-renewal and tumorigenicity of SCs/CSCs. Although the precise role of S100A4 in Notch2, and PTEN-PI3K-Akt signaling within cancer cells remains to be elucidated, we are the first group to show that S100A4 regulates Notch2, and PTEN-PI3K-Akt expression. We further extended findings by Harris MA et al that S100A4 is significantly up-regulated not only in glioma CSCs cells (20) but also in HN-CICs. Together, all these findings highlight the importance of aberrant expression of S100A4 in neoplastic process, and up-regulation of S100A4 plays an important role in CSCs theory.

We found more nuclear and cytoplasmic staining of S100A4 in the moderate to
poor-differentiated HNSCC tissues (Fig. 5A). Grigorian et al and Fernandez-Fernandez et al have demonstrated that S100A4 directly interacts with P53 after the binding of Ca\(^{2+}\) (44, 45).

Lin et al report that \(P53\) negatively regulates the transcriptional activity of stem cell marker, Nanog (46). We also found that the S100A4 promoter was most hyper-methylated in HNSCCs but hypo-methylated in our enriched HN-CICs (data not shown). Therefore, our current hypothesis is that epigenetic modifications of the promoter region of S100A4 gene regulates S100A4 expression; consequently, S100A4 plus Ca\(^{2+}\) abrogates the negative regulation of P53 on Nanog to enhance the expression of Nanog. Overall, future research delineates the details of how S100A4 regulates its downstream targets and how these interactions influence the stemness properties of CSC remains to be determined.

As being a known CIC markers of HNSCC (47), it was also important to acknowledge the relative position of \(CD44\) in the EMT-Calcium-ESC networks. \(CD44\), ranked the 104th
cut-node (out of 208) in the Ca\textsuperscript{2+}-EMT networks, was connected with the \textit{EGFR}, \textit{MMP1}, and \textit{VCAN} in the \textit{1st} and \textit{2nd}-order connecting subnetworks. However, we did not find significant changes of \textit{CD44} after S100A4 knockdown. We speculated that inconsistent trends between different splice variants of \textit{CD44} (48) or alternative routing in calcium signaling pathways, different from S100A4, might be possible explanations (49). Further research effort is needed in this area.

Together, our present research showed that the S100A4 signaling pathways play a major role in the maintenance of HN-CICs population and targeting S100A4 signaling might be a potential therapeutic target for HNSCC by eliminating CICs. In addition, expression of S100A4 should be a useful prognostic factor for HNSCC patients.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Figure Legends

Figure 1. Expression profiles of EMT-related genes in enriched HN-CICs. The differential transcriptome profiles between SAS cells and SAS-derived HN-CICs under 2, 3, 5 or 9 weeks of cultivation with defined serum-free selection medium were collected and analyzed. A, Cluster 4 differential gene expression profile. B, The heat maps of the Cluster 4 transcripts. Red and blue indicate high and low expression levels, respectively. C, Functional annotation of Cluster 4. D, The expression of S100A4 transcript in parental HNSCCs (SAS and OECM1) or derived HN-CICs was detected by real-time RT-PCR analysis (Data were means ± SD of triplicate samples from three experiments) (left panel). Protein level of S100A4 and GAPDH in parental HNSCCs or HN-CICs cells were analyzed by immunoblotting (middle panel). Intracellular localization of S100A4 of enriched HN-CICs was examined by immunofluorescence. Magnification, X200 (right panel).
Figure 2. Depletion of S100A4 impairs self-renewal and stemness properties but enhances cell differentiation and apoptosis of HN-CICs. A, Protein level of S100A4 in S100A4 stable knockdown (Sh-S100A4-1 or Sh-S100A4-2) HNSCCs (SAS and primary HNSCC) was detected by western blotting (left upper and lower panels). S100A4 stable knockdown HNSCCs were grown under defined serum-free selection medium for primary spheres formation, then, serial passage spheres, including secondary sphere and tertiary sphere established from primary sphere after every 3 weeks of incubation, were generated. The numbers of primary spheres, secondary spheres and tertiary spheres of SAS or primary HNSCC cells with S100A4 stable knockdown were calculated, respectively (right upper and lower panels). B, The ALDH enzymatic activity of Sh-S100A4 and control (Sh-Luc) HNSCCs (SAS and primary HNSCC) were examined. Side population cells (SPs) of primary
HNSCC *(middle panel)* or OECM1 *(right panel)* in Sh-S100A4 and control OECM1 cells were examined, respectively. C, SAS or primary HNSCC-derived HN-CICs infected with Sh-S100A4-1, Sh-S100A4-2 or Sh-Luc lentivirus were further cultivated under the serum-free defined selection medium, and the cellular morphology of virus infected cells were observed. Arrows indicated the attached epithelial-like cells. D, Representative expression profile of CK18 in HN-CICs (SAS, *left panel* and primary HNSCC, *middle panel*) cells infected with Sh-Luc, or Sh-S100A4-1 lentivirus was assessed by FACS. Single cell from figure-2D primary HNSCC cells was stained with Annexin V *(right panel)*. Results are means ± SD of triplicate samples from three experiments.

**Figure 3. Knockdown of S100A4 attenuates HNSCC xenograft tumor growth.** A, Summary of the *in vivo* tumor growth ability of S100A4 stable knockdown and Sh-Luc SAS
sells examined by xenotransplantation.  

B, Representative tumor growth of control SAS cells (SAS-Luc: 2.5×10⁵) or S100A4-knockdown SAS cells (*upper panels*). Tumor volume was measured after inoculation of Sh-S100A4-1 or sh-Luc–expressing cells. Error bars correspond to SD (*middle panel*). The survival curves of mice injected with the Sh-S100A4-1 cells (solid circle with solid line) or Sh-Luc cells (open circle with dash line) were recorded (*bottom panel*).  

C, Parental SAS cells (5×10⁵ cells) were subcutaneously implanted into left and right back of nude mice and allowed to develop tumors to a size around 0.2 cm³ (12 days). On days 12, 15, 20, and 25 after implantation, progressively growing SAS-derived tumors were subject to Sh-Luc or Sh-S100A4-1 lentivirus as arrows indicated and recorded, respectively. A representative result of tumor growth on nude mice; red fluorescence indicated the RFP reporter signal (*upper panels*). Tumor volume was measured after inoculation of parental SAS cells and injection of lentiviruses expressing control viruses or
Sh-S100A4-1 lentivirus. Error bars correspond to SD (middle panel). The survival curves of tumor-bearing nude mice treated with the Sh-S100A4-1 or Sh-Luc lentivirus were recorded (bottom panel).

**Figure 4.** Overexpression of S100A4 enhances expression of EMT markers, stemness properties and in vitro tumorigenic potentials of HNSSCs. **A,** Total proteins were prepared from control or S100A4-overexpressing HNSCCs, and analyzed by immunoblotting against anti-E-cadherin, anti-Vimentin or anti-GAPDH antibodies as indicated. **B,** Total proteins of control or S100A4-overexpressing HNSCCs, afterward 2 weeks of cultivation under serum-free defined medium, were isolated and immunoblotted against anti-Oct-4 and anti-Nanog (right panel). **C,** The tumor spheres formation efficiency of control-GFP and S100A4-overexpressing HNSCCs (SAS and OECM1) under serum-free defined medium for
1, 2, 3 or 4-week, were calculated. D. Side population cells of stable S100A4-overexpressing and control Sh-GFP–expressing HNSCCs were analyzed (right panel). Results are means ± SD of triplicate samples from three representative experiments.

Figure 5. Correlation of S100A4 expression to clinical grading, predicted survival rate and stemness markers of HNSCC patients. A, Representative results of immunohistochemistry staining of S100A4 on HNSCC patients with different stages were shown. Arrows indicated the positive staining of S100A4 (black arrows: cytoplasmic staining; yellow arrows: nucleus staining). Magnification was shown at lower right corner. B, Table of S100A4 expression and clinicopathologic variables in 102 HNSCC patients. C, Kaplan-Meier analysis of overall survival (left panel) and recurrence status (right panel) in 102 HNSCC patients according to the expression of S100A4 (-, 0-10% S100A4 positive cells; +, 10-50%
S100A4 positive cells; ++, more than 50% S100A4 positive cells) (*, P<0.05; **, p<0.01). D,

Co-expression profiles between S100A4 and Nanog (left panel), Oct-4 (middle panel) or CD133 (right panel) of 34 HNSCC patients was examined immunohistochemically.

Figure 6. S100A4 knockdown affects molecular mechanisms involved in developmental (Notch2) and cell survival (PTEN-PI3K-Akt) signaling pathways. A, Major subnetwork of 1st and 2nd-order neighbors of significantly perturbed genes after shRNAi-mediated knockdown of S100A4. Node coloring indicates gene activities – red, induced, and green, suppressed; node size, number of neighbor genes; node border and edge coloring, gene sets – pink, ESC, blue, EMT-Calcium, and orange, both. Neighbor genes of S100A4 were illustrated in the “inset”. B, Total proteins from Sh-Luc and Sh-S100A4 expressing HNSCCs were prepared and analyzed by immunoblotted with anti-Notch-2, anti-PTEN, anti-pAKT,
anti-PI3K or anti-GAPDH antibodies as indicated.
**Figure 1, Lo, JF et al.**

A) Cluster 4

B) SAS derived HN-CICs

C)

<table>
<thead>
<tr>
<th>Cluster</th>
<th>Number of probes</th>
<th>Functional annotation</th>
<th>Geometric mean of ( P )-values</th>
<th>Modified fisher exact ( P )-value</th>
</tr>
</thead>
</table>
| 4       | 25               | GO:0001837~epithelial to mesenchymal transition  
|         |                  | GO:0048762~mesenchymal cell differentiation  
|         |                  | GO:0014031~mesenchymal cell development  
|         |                  | GO:0048468~cell development  | 3.45E-4  | 6.02E-09  |
|         |                  |                        |                               | 7.42E-08  | 7.42E-08  |
|         |                  |                        |                               | 0.002571  |

D) S100A4 immunofluorescence
Figure 2, Lo, JF et al.

A

SAS

Sh-Luc
Sh-S100A4-1
Sh-S100A4-2

Primary HNSCC

Sh-Luc
Sh-S100A4-1
Sh-S100A4-2

S100A4
GAPDH

S100A4
GAPDH

B

SAS

Primary HNSCC

ALDH1+ activity %

Primary HNSCC

ALDH1+ activity %

C

SAS-derived spheres

Primary HNSCC-derived spheres

Sh-Luc
Sh-S100A4-1
Sh-S100A4-2

200X

200X

200X

200X

D

Primary HNSCC

Primary HNSCC

CK18 positive (%)

Annexin V positive (%)

Control-IgG
SAS-Sh-Luc
SAS-Sh-S100A4-1
SAS-Sh-S100A4-2

60 %

CK18

Control-IgG
SAS-Sh-Luc
SAS-Sh-S100A4-1
SAS-Sh-S100A4-2

0

10

20

30

0

10

20

30

40

50

**Figure 3, Lo, JF et al.**

A

| Tumorigenicity of SAS-Sh-Luc and SAS-Sh-S100A4 Cells in Nude Xenotransplant Assay |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| **Cell Numbers for Injection**  | **2x10^6**      | **1x10^6**      | **5x10^5**      | **2.5x10^5**    |
| SAS-Sh-Luc                      | 6/6             | 6/6             | 6/6             | 6/6             |
| SAS-Sh-S100A4-1                 | 2/6             | 2/6             | 1/6             | 0/6             |

B

**Lentivirus infected SAS cells**

C

**SAS derived tumors treated with lentiviral Sh-RNAi**

**SAS-Sh-RNAi**

(▲, △) Sh-Luc
(□, ○) Sh-S100A4-1

**SAS-derived xenografts**

![Graph showing tumor volume over time for Sh-Luc and Sh-S100A4-1](image)

Days after injection: 0, 5, 8, 12, 15, 20, 25, 28

Tumor volume (cm^3)

* P<0.05

**Survival %**

Sh-Luc
Sh-S100A4-1

weeks: 0, 2, 4, 6, 8, 10, 12, 14, 16

* * P<0.01
Figure 4, Lo, JF et al.

A

<table>
<thead>
<tr>
<th>SAS</th>
<th>OECM1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>S100A4</td>
</tr>
<tr>
<td>Control</td>
<td>S100A4</td>
</tr>
<tr>
<td>S100A4</td>
<td>S100A4</td>
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<td>S100A4</td>
</tr>
<tr>
<td>S100A4</td>
<td>S100A4</td>
</tr>
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</table>

B

![Graph showing Tumor spheres/well over weeks](image)

C

<table>
<thead>
<tr>
<th>SAS</th>
<th>OECM1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>S100A4</td>
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<td>S100A4</td>
<td>S100A4</td>
</tr>
</tbody>
</table>

D

![Graph showing SP cells % over SAS and OECM1](image)
Figure 5, Lo, JF et al.

A

Hyperplasia  
Well-Differentiated  
Moderate-Differentiated  
 Poor-Differentiated  
Lymph node metastasis

B

Table. S100A4 expression and clinicopathologic variables

<table>
<thead>
<tr>
<th>Variables</th>
<th>(n=102)</th>
<th>- (%)</th>
<th>+ (%)</th>
<th>++ (%)</th>
<th>P Value</th>
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</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥ 54</td>
<td>38</td>
<td>9 (24)</td>
<td>18 (47)</td>
<td>11 (29)</td>
<td>0.472</td>
</tr>
<tr>
<td>&lt; 54</td>
<td>64</td>
<td>21 (33)</td>
<td>23 (36)</td>
<td>20 (31)</td>
<td></td>
</tr>
<tr>
<td>Differentiation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well</td>
<td>39</td>
<td>18 (46)</td>
<td>19 (49)</td>
<td>2 (5)</td>
<td>***P&lt;0.0001</td>
</tr>
<tr>
<td>Moderate to Poor</td>
<td>63</td>
<td>12 (19)</td>
<td>22 (35)</td>
<td>29 (46)</td>
<td></td>
</tr>
<tr>
<td>T - stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precancer - II</td>
<td>29</td>
<td>18 (62)</td>
<td>6 (21)</td>
<td>5 (17)</td>
<td>***P&lt;0.0001</td>
</tr>
<tr>
<td>T3 - T4</td>
<td>73</td>
<td>12 (16)</td>
<td>35 (48)</td>
<td>26 (36)</td>
<td></td>
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<tr>
<td>Lymph node Metastasis</td>
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</tr>
<tr>
<td>N = 0</td>
<td>57</td>
<td>29 (51)</td>
<td>28 (49)</td>
<td>0 (0)</td>
<td>***P&lt;0.0001</td>
</tr>
<tr>
<td>N ≥ 1</td>
<td>45</td>
<td>1 (2)</td>
<td>13 (29)</td>
<td>31 (69)</td>
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<tr>
<td>Stage</td>
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<tr>
<td>Precancer - II</td>
<td>20</td>
<td>16 (80)</td>
<td>4 (20)</td>
<td>0 (0)</td>
<td>***P&lt;0.0001</td>
</tr>
<tr>
<td>III - IV</td>
<td>82</td>
<td>14 (17)</td>
<td>37 (45)</td>
<td>31 (38)</td>
<td></td>
</tr>
</tbody>
</table>

* Chi-Square test. (-, 0-10% positive cells; +, 10-50% positive cells; more than 50% positive cells)

C

Probability of survival(%)  
0 vs. 2: ** P=0.0017  
1 vs. 3: ** P=0.0001  
2 vs. 3: P=0.221

Recurrence-free (%)  
0 vs. 2: ** P=0.0004  
1 vs. 3: ** P=0.001  
2 vs. 3: P=0.331

D

<table>
<thead>
<tr>
<th>S100A4</th>
<th>Nanog</th>
<th>Negative</th>
<th>Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group1</td>
<td>23%(8/34)</td>
<td>9%(3/34)</td>
<td></td>
</tr>
<tr>
<td>Group2</td>
<td>9%(3/34)</td>
<td>59%(20/34)</td>
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* P=0.138 Fisher extract test

<table>
<thead>
<tr>
<th>S100A4</th>
<th>Oct-4</th>
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<th>Positive</th>
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<tbody>
<tr>
<td>Group1</td>
<td>23%(8/34)</td>
<td>9%(3/34)</td>
<td></td>
</tr>
<tr>
<td>Group2</td>
<td>12%(4/34)</td>
<td>56%(19/34)</td>
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</table>

* ***P=0.001 Fisher extract test

<table>
<thead>
<tr>
<th>S100A4</th>
<th>CD133</th>
<th>Negative</th>
<th>Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group1</td>
<td>21%(7/34)</td>
<td>15%(5/34)</td>
<td></td>
</tr>
<tr>
<td>Group2</td>
<td>18%(6/34)</td>
<td>46%(16/34)</td>
<td></td>
</tr>
</tbody>
</table>

* ***P=0.005 Fisher extract test

P=0.138 Fisher extract test
Figure 6, Lo, JF et al.
The Epithelial-Mesenchymal Transition Mediator S100A4 Maintains Cancer Initiating Cells in Head and Neck Cancers

Jeng-Fan Lo, Cheng-Chia Yu, Shih-Hwa Chiou, et al.

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