Research Paper

Epigenetic modification of *SALL1* as a novel biomarker for the prognosis of early stage head and neck cancer

Kiyoshi Misawa[1][✉], Yuki Misawa[1], Atsushi Imai[1], Daiki Mochizuki[1], Shiori Endo[1], Masato Mima[1], Ryuji Ishikawa[1], Hideya Kawasaki[2], Takashi Yamatodani[1], and Takeharu Kanazawa[3]

1. Department of Otolaryngology/Head and Neck Surgery, Hamamatsu University School of Medicine, Hamamatsu, Shizuoka, Japan
2. Department of Regenerative & Infectious Pathology, Hamamatsu University School of Medicine, Hamamatsu, Japan
3. Department of Otolaryngology/Head and Neck Surgery, Jichi Medical University, Tochigi, Japan

✉ Corresponding author: Kiyoshi Misawa, Department of Otolaryngology/Head and Neck Surgery, Hamamatsu University School of Medicine, 1-20-1 Handayama, Hamamatsu, Shizuoka, Japan, 431-3192. Phone: 81-53-435-2252, fax: 81-53-435-2253, email: kiyoshim@hama-med.ac.jp

Abstract

This study examined *Sal-like protein (SALL)1* methylation profiles in head and neck squamous-cell carcinoma (HNSCC) patients at diagnosis and follow-up, and evaluated their prognostic significance and value as a biomarker. *SALL1* expression was examined in a panel of cell lines by quantitative reverse transcription PCR (qRT-PCR). Promoter methylation was determined by quantitative methylation-specific polymerase chain reaction (qMSP) and was compared to the clinical characteristics of 205 samples. *SALL1* promoter methylation was associated with transcriptional inhibition and was correlated with disease recurrence in 31.7% of cases, with an odds ratio of 1.694 (95% confidence interval: 1.093–2.626; P = 0.018) by multivariate Cox proportional hazard regression analysis. *SALL1* promoter hypermethylation showed highly discriminatory receiver operator characteristic curve profiles that clearly distinguished HNSCC from adjacent normal mucosal tissue, and was correlated with reduced disease-free survival in early stage T1 and T2 patients (log-rank test, P < 0.001). *SALL1* methylation was significantly correlated with the methylation status of both *SALL3* and *CDH1*. This study suggests that CpG hypermethylation is a likely mechanism of *SALL1* gene inactivation, supporting the hypothesis that *SALL1* might play a role in HNSCC tumorigenesis and could serve as an important biomarker.

Key words: SALL1, methylation, early tumor stage patients, biomarker, HNSCC

Introduction

Head and neck squamous-cell carcinoma (HNSCC) is the sixth most frequently occurring type of cancer in the world [1]. It is a highly heterogeneous disease that develops through one of two primary mechanisms: chemical carcinogenesis through exposure to tobacco and alcohol or virus-induced tumorigenesis [2]. Surgical intervention is challenging for HNSCC, as this disease is associated with a limited surgical margin, as tumors are located close to vital tissues such as the central nervous system, carotid artery, trachea, and esophagus. In addition, despite aggressive, site-specific multimodal therapy, a significant proportion of patients will develop disease recurrence, with a risk of local failure of up to a 60% and a risk of distant failure of up to a 30% [3, 4]. To improve the survival rate of HNSCC patients, it is

necessary to identify reliable biomarkers that provide sufficient prognostic power for the effective clinical management of this disease.

The *spalt (Sal)* genes were first identified in *Drosophila*. Mice and humans have four known *Sal*-related genes named *sall1* to *sall4* and *SALL1* to *SALL4*, respectively [5]. Heterozygous early-truncating *SALL1* is known to cause autosomal dominant Townes–Brocks syndrome, a malformation syndrome, without severe central nervous system involvement [6]. Recent studies have investigated the association between *SALL1* expression and carcinogenesis. One group demonstrated that the inhibition of *SALL1* correlates with reduced levels of *CDH1*, an important contributor to epithelial-to-mesenchymal transition [7]. Another report showed that *SALL1*

hypermethylation resulted in reduced levels of *SALL1* mRNA in breast carcinoma [8], and aberrant hypermethylation of *SALL1* was found to contribute to carcinogenesis in chronic lymphocytic leukemia [9]. Despite accumulating evidence implicating this event in adenocarcinomas, the hypermethylation of *SALL1* in SCCs of tissues such as head and neck, esophagus, lung, and cervix, needs to be explored.

We demonstrate that loss of *SALL1* expression is associated with hypermethylation of key CpG sites within transcription factor binding domains and that expression can be restored after treatment with the demethylating agent, 5-azacytidine. Furthermore, assessment of primary tumor specimens confirmed that hypermethylation is common in in-patient tumors and is directly associated with recurrence. This study suggests that hypermethylation of *SALL1* in primary tumors is an independent predictor of survival for head and neck cancer.

Materials and Methods

Tumor samples and cell lines

A total of 205 primary HNSCC specimens were obtained during surgery at the Department of Otolaryngology of Hamamatsu University School of Medicine. Clinical information including age, gender, tumor location, smoking status, alcohol consumption, tumor size, lymph node status, and tumor stage was obtained from clinical records. The mean patient age was 64.8 years (range: 32–90 years), and the male:female ratio was 175:30. Primary tumors were located in the hypopharynx (n = 51), larynx (n = 40), oropharynx (n = 51), and oral cavity (n = 63). Patients provided written, informed consent for participation in the study and the protocol was approved by the Institutional Review Boards at the Hamamatsu University School of Medicine. Normal tonsillar specimens, Ton7, Ton8, and Ton10, were surgically obtained from patients with chronic tonsillitis. DNA and cDNA from eight UM-SCC cell lines, MJF, BDF, 81F, 99F fibroblasts cell lines were provided by Dr. Thomas E. Carey of the University of Michigan. Normal human keratinocytes were a gift from Dr. No Hee Park of the University of California at Los Angeles School of Dentistry [10]. For reactivation of *SALL1* expression, cultures were incubated for 48 h with 5-azacytidine (15 μg/mL, Sigma-Aldrich, St. Louis, MO, USA), a DNA methyltransferase inhibitor [11].

Bisulfite modification and quantitative methylation-specific polymerase chain reaction (qMSP)

Extraction and bisulfite conversion of genomic DNA was carried out as previously described [12][13].

SALL1 promoter methylation was assessed by qMSP with the TP800 Thermal Cycler Dice Real-Time System (Takara Bio, Otsu, Japan) using the primer sequences shown in Supplementary Table S1. *SALL1* primers amplified sequences upstream of, around, and downstream of the transcription start site. A standard curve was generated using serial dilutions of EpiScope Methylated HeLa genomic DNA (Takara Bio, Otsu, Japan), with fully methylated (FM) DNA used as a control. Normalized methylation values (NMV) was defined as follows: NMV = (*SALL1*-S/*SALL1*-FM)/(*ACTB*-S/*ACTB*-FM), where *SALL1*-S and *SALL1*-FM represent *SALL1* methylation levels in the sample and universally methylated DNA, respectively, and *ACTB*-S and *ACTB*-FM correspond to β-actin in the sample and the universally methylated DNA, respectively [14]. To analyze the methylation status of *SALL3* [15], *CDH1* [16], *RASSF1A* [16], *p16* [16], *MGMT* [17], *DAPK* [17], *DCC* [18], *CDH13* [19], and *COL1A2* [20] primers and conditions were used as previously described.

RNA extraction and quantitative reverse transcription-PCR (qRT-PCR)

Total RNA was isolated with the RNeasy Plus Mini kit (Qiagen, Valencia, CA, USA), and cDNA was synthesized with the ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan) [14]. Primer sequences are shown in Supplementary Table S1. Target mRNA expression was compared between samples by normalization to *glyceraldehyde 1-phosphate dehydrogenase* (*GAPDH*) mRNA expression.

The Cancer Genome Atlas (TCGA) data

Aberrant DNA methylation data from the TCGA (available in June, 2017) was collected from MethHC, a database of DNA methylation and gene expression in human cancer (http://methhc.mbc.nctu.edu.tw /php/index.php) [21]. The DNA methylation data were collected using the Infinium HumanMethyla-tion450 platform (Illumina, Inc., San Diego, CA, USA) and are presented as β values.

Data analysis and statistics

Receiver-operator characteristic ROC curve analysis was performed using the NMVs for 36 HNSCC and 36 adjacent normal mucosal samples and the Stata/SE 13.0 system (Stata Corporation, TX, USA). The area under the ROC curve indicated the optimal sensitivity and specificity cut-off levels for distinguishing between methylation levels in normal and HNSCC tissue, and the NMV thresholds were calculated for each target gene. The cut-off values were used to determine the methylation frequencies of the *SALL1* genes.

Associations between variables were assessed by performing a Student's *t*-test and Pearson's chi-square test. Disease-free survival (DFS) was measured from the date the initial treatment to the date of diagnosis of locoregional recurrence or distant metastasis. The Kaplan-Meier test was used to calculate survival probabilities and the log-rank test was used to compare survival rates. The prognostic value of methylation status was assessed by performing a multivariate Cox proportional hazards analysis adjusted for age (≥ 70 versus < 70 years), gender, alcohol intake, smoking status, and tumor stage (I–III versus IV). Differences with P < 0.05 were considered significant. All statistical analyses were performed using StatMate IV software (ATMS Co., Ltd., Tokyo, Japan).

Results

Initial Screening: Matched Pairs of Head and Neck Tumors and Adjacent Normal Mucosal Tissues

SALL1 CpG islands and regions, analyzed by qMSP, are shown in Fig. 1A. Initially, the methylation status of the *SALL1* promoter was analyzed in 36 cancerous and paired noncancerous mucosae by qMSP. Promoter methylation levels were represented by NMVs, which is the ratio of methylated DNA at the target sequence in each specimen to a fully methylated control DNA. The *SALL1* methylation level was significantly higher in primary HNSCCs than in noncancerous mucosal tissues (median NMV = 0.194 versus 0.025, P = 0.020; paired Student's *t*-test; Fig. 1B). *SALL1* promoter hypermethylation exhibited highly discriminative ROC curve profiles, which clearly distinguished HNSCC from normal mucosal tissues (area under the ROC = 0.730). At the cutoff value of 0.036, sensitivity was 58.3% and specificity was 88.9% (Fig. 1C).

SALL1 methylation status in tumor samples and cancer cell lines

Among the 205 DNA samples obtained from the examined untreated primary tumors, a specimen was classified as methylated when the NMV exceeded 0.036. The *SALL1* promoter was methylated in 65/205 (31.7%) of cases and unmethylated in 140/205 (68.3%) of cases (Fig. 1D). Matched pairs of head and neck tumors and adjacent normal mucosal tissues were obtained from surgical specimens collected from 96 patients for methylation screening. These data are presented as Supplementary Table S2. qMSP indicated a significantly increased NMV for *SALL1* promoter methylation in cancer cell lines versus that in normal cell lines and normal tonsillar tissues (P = 0.010 and P = 0.011, respectively; Fig. 1E).

SALL1 expression analysis by qRT-PCR

The levels of *SALL1* mRNA were determined in 89 HNSCC tissues and adjacent normal mucosal tissues using qRT-PCR. The *SALL1* mRNA level in HNSCC tissues was found to be 6.9-fold lower compared to that in the paired non-cancerous mucosa (P < 0.001; Fig. 2A). The levels of *SALL1* mRNA were significantly decreased in cases in which the *SALL1* promoter region was methylated (P = 0.045; Fig. 2B). qRT-PCR of *SALL1* transcripts from 10 UM-SCC cell lines revealed lower expression in cancer cell lines than in normal fibroblast cell lines (P < 0.001; Fig. 2C). We treated the three silenced cell lines (UM-SCC-2, UM-SCC-6, and UM-SCC-54) with a DNA methyltransferase inhibitor (5-azacytidine), and all three cell lines exhibited restored *SALL1* expression after inhibition of methyltransferase activity (Fig. 2D).

SALL1 promoter methylation correlates with clinicopathologic parameters and predicts poor patient outcome

Age, gender, smoking status, alcohol intake, tumor size, lymph node status, and clinical stage were not associated with *SALL1* promoter methylation status in 205 patients. However, *SALL1* methylation was significantly correlated with recurrence events, *SALL3* methylation status, and *CDH1* methylation status (P < 0.001, P = 0.003, and P < 0.001, respectively). No other molecular markers were significantly correlated with *SALL1* methylation status (Table 1, Supplementary Table S3).

Kaplan–Meier plots revealed that methylation of the *SALL1* gene in 205 tumors were related to DFS in some patients (log-rank test, P < 0.001; Fig. 3A). Patients with T1 and T2, negative lymph node metastasis, positive lymph node metastasis, and Stage I, II, and III disease had decreased DFS compared to those without *SALL1* methylation (P < 0.001 P = 0.029, P < 0.001, and P < 0.001, respectively; Fig. 3B–F). *SALL1* methylation status in patients with T3 and T4 and Stage IV tumors was not related to outcome (Fig. 3C, G). In addition, results based on multivariate Cox proportional hazard regression analysis, which included age, gender, alcohol consumption, smoking status, and tumor stage, indicated that survival rates were 1.694-fold (95% CI, 1.093–2.626) lower in patients with *SALL1* gene methylation than in those without methylation (P = 0.018; Table 2). Moreover, based on 98 patients with T1 and T2 tumor stages, the group with methylated *SALL1* had a 2.602-fold greater hazard (95% CI, 1.296–5.224) than the group without methylation (P = 0.007; Table 3). When both *SALL1* and *SALL3* were methylated in the tumor, there was a significantly lower rate of DFS than if *SALL1* and *SALL3* were unmethylated (log-rank test, P = 0.012)

(Supplementary Fig. S1A). Concurrent hypermethylation of *SALL1* and *CDH1* promoters was also significantly correlated with reduced DFS (log-rank test, P = 0.005; Supplementary Fig. S1B).
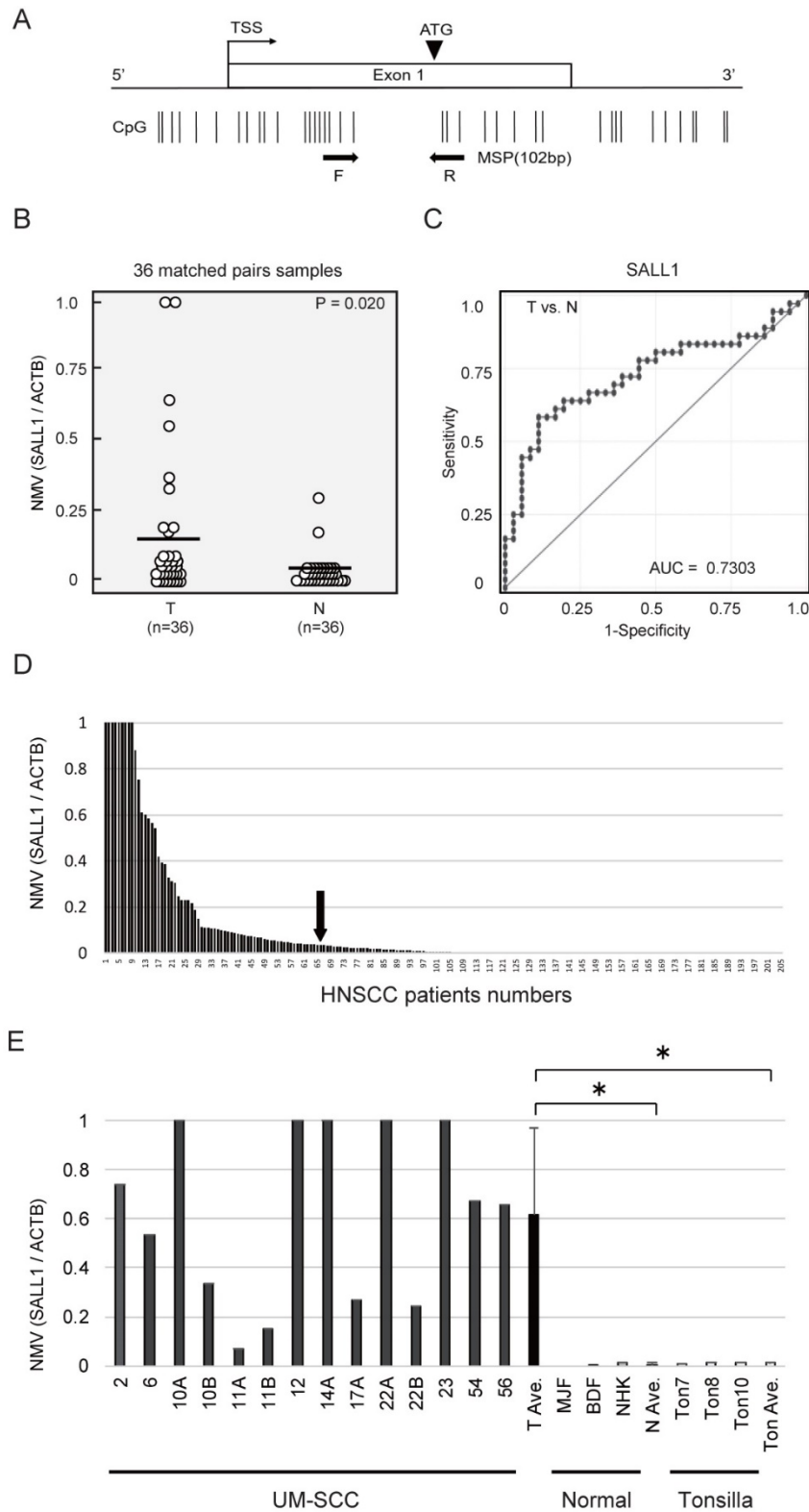

Figure 1. Schematic representation of *SALL1* gene methylation analysis by quantitative methylation-specific polymerase chain reaction (qMSP) and evaluation of *SALL1* methylation in head and neck squamous-cell carcinoma (HNSCC) patients and UM-SCC cell lines. (**A**) *SALL1* exon structure and CpG sites within expanded views of the promoter region relative to the transcript start site (TSS). Vertical lines, individual CpG sites. Straight arrows, relative location of the primers used for qMSP; bent arrow, TSS; arrowhead, translation start site (ATG). Straight arrows, relative location of the forward (F) and reverse (R) primers used for qMSP. (**B**) *SALL1* normalized methylation values (NMVs) of head and neck tumors were higher than those of adjacent normal mucosal tissues (P = 0.020). (**C**) AUROC value for *SALL1* was 0.7303. At the cutoff value of 0.036, sensitivity was 58.3% and specificity was 88.9%. (**D**) Two hundred and five DNA samples from untreated primary tumors were tested with qMSP. The cutoff NMVs (0.036) are indicated by straight arrows. (**E**) Representative examples of qMSP of *SALL1* in eight UM-SCC cell lines, fibroblasts (MJF and BDF), keratinocytes (NHK), and normal tonsillar tissues (Ton7, Ton8, and Ton10). * P < 0.01.
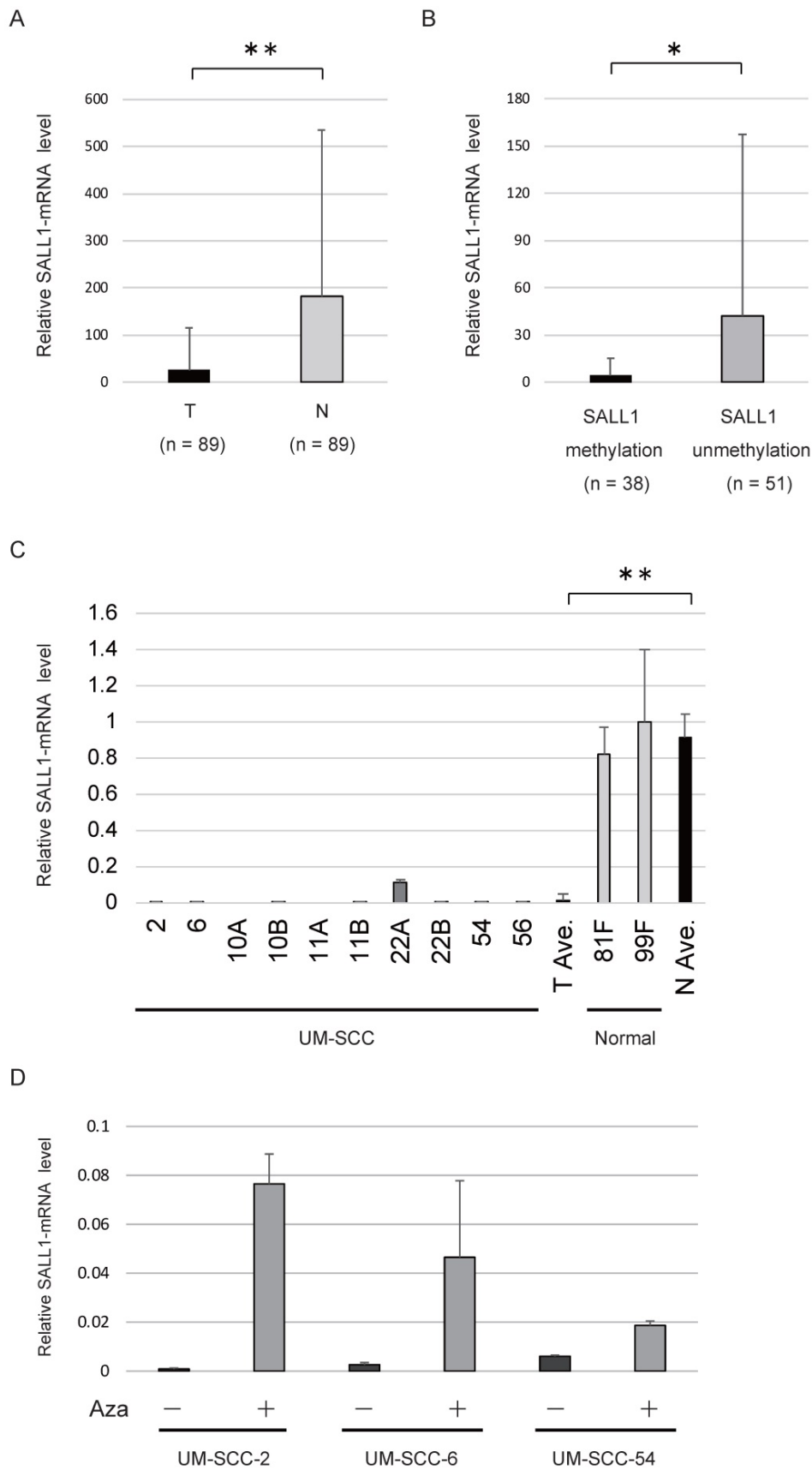
Figure 2. *SALL1* expression as assessed by quantitative reverse transcription PCR (qRT-PCR). (**A**) Relative mRNA expression level of *SALL1* in 89 matched pairs of head and neck tumors and adjacent normal mucosal tissues. T, tumor samples; N, normal samples. ** P < 0.01. (**B**) *SALL1* mRNA expression was inversely correlated with *SALL1* hypermethylation. * P < 0.05. (**C**) Relative mRNA expression level of *SALL1*, as determined by qRT-PCR in 10 UM-SCC and two normal cell lines. ** P < 0.01. (**D**) Effect of 5-azacytidine (Aza) on *SALL1* expression in three cell lines with densely methylated *SALL1*, as evaluated by qRT-PCR. Controls were cells that were similarly treated, but without Aza.
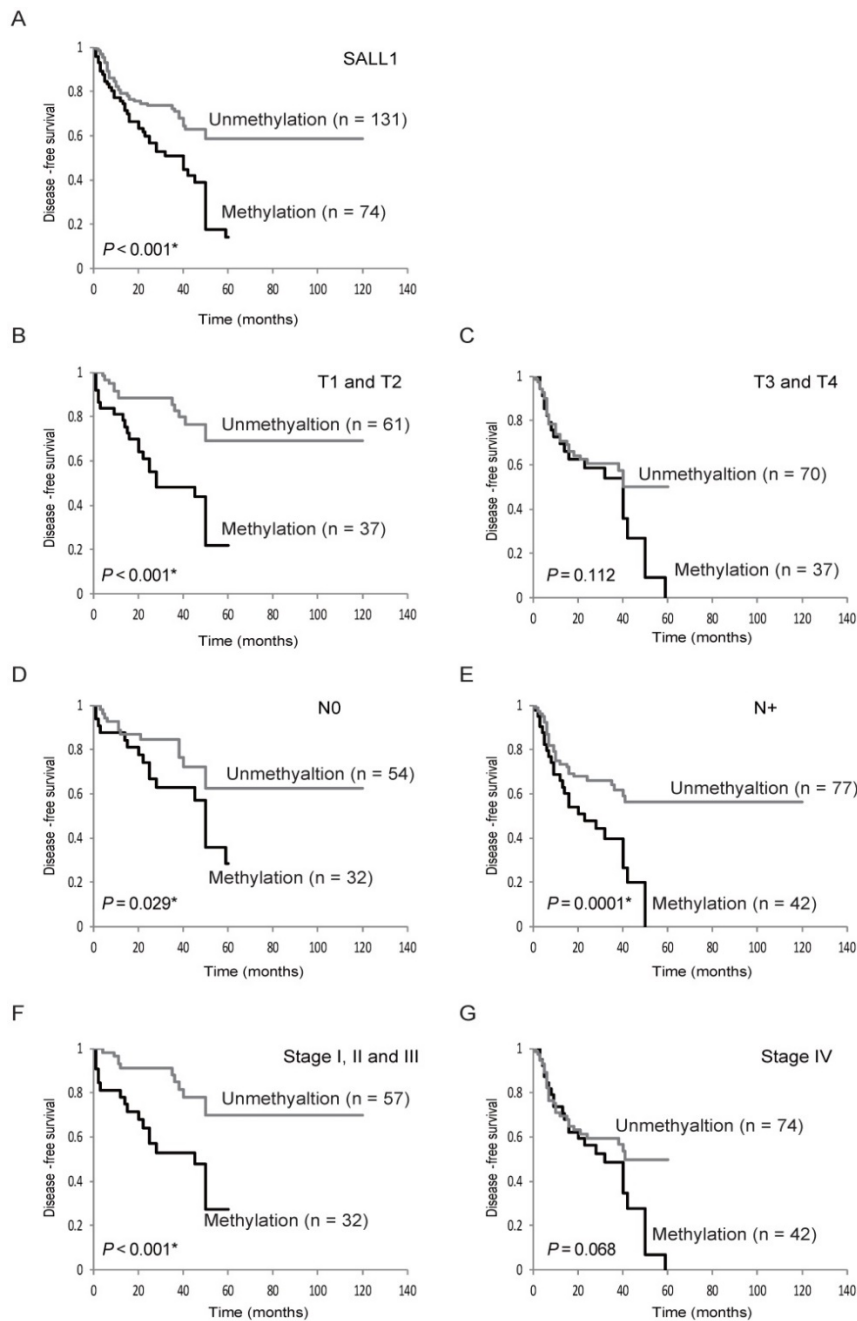
Figure 3. Kaplan-Meier survival curves for head and neck squamous-cell carcinoma (HNSCC) patients. Disease-free survival (DFS) for **(A)** all 205 HNSCC cases, **(B)** tumor size in T1 and T2 cases (n = 98), **(C)** tumor size in T3 and T4 cases (n = 107), **(D)** lymph node status in N0 cases (n = 86), **(E)** lymph node status in N+ cases (n = 119), **(F)** stage I, II, and III cases (n = 89), and **(G)** stage IV cases (n = 116). Gray and black lines indicate patients with tumors without and with methylation, respectively.

External validation of The Cancer Genome Atlas (TCGA) data

HNSCC data from TCGA were examined for *SALL1* DNA methylation. *SALL1* methylation showed an average β value of 0.381 in the HNSCC TCGA cohort as compared to 0.129 in normal samples (P < 0.001, Student's *t*-test; Supplementary Fig. S2).

Discussion

Clarifying the epigenetic regulation of *SALL1* can provide insights into the mechanisms of tumorigenesis and the risk of disease recurrence for various tumor types. The present study investigated *SALL1* promoter methylation profiles in 205 HNSCC patient tissues. We found that hypermethylation of CpG islands in the *SALL1* promoter was independently associated with early T1 and T2 tumor behavior, suggesting that *SALL1* acts as a tumor suppressor gene and can serve as a prognostic biomarker in HNSCC.

Table 1. SALL1 Gene Methylation Status in Primary Samples of HNSCC in 205 patients

Patient and tumor characteristics (n=205)	Methylation		P-value†
	Present (n=74)	Absent (n=131)	
Age			
70 and older (67)	24	43	
Under 70 (138)	50	88	1
Gender			
Male (175)	62	113	
Female (30)	12	18	1
Smoking status			
Smoker (157)	55	102	
Nonsmoker (48)	19	29	1
Alcohol intake			
Ever (149)	55	94	
Never (56)	19	37	0.746
Tumor size			
T1, 2 (98)	37	61	
T3, 4 (107)	37	70	1
Lympho-node status			
N0 (86)	32	54	
N+ (119)	42	77	1
Stage			
I, II, III (89)	32	57	
IV (116)	42	74	1
Recurrence events			
Positive (85)	43	42	
Negative (120)	31	89	< 0.001*

† Fisher's exact probability test.

* P < 0.05.

Table 2. Multivariate analysis of factors affecting survival using Cox proportional hazards model in 205 patients

Variables	Disease-free survival	
	HR (95% CI)	P-value
Age		
70 and older vs. <70	1.100 (0.685-1.752)	0.702
Sex		
Male vs. Female	1.164 (0.627-2.160)	0.631
Smoking status		
Smoker vs. Non smoker	1.990 (0.259-15.28)	0.508
Alcohol exposure		
Ever vs. Never	1.234 (0.738-2.065)	0.423
Stage		
I, II, III vs. IV	2.042 (1.284-3.250)	0.003*
SALL1 methylation		
Yes vs. No	1.694 (1.093-2.626)	0.018*

HR: hazard ratio.

95% CI: 95% confidence interval.

* P < 0.05.

SALL1 (16q12.1) has been associated with Townes-Brocks Syndrome, a disorder characterized by multiorgan dysgenesis including renal, heart, and genital malformation [22, 23]. Moreover, targeted deletion of mouse *Sall1* results in kidney agenesis or severe dysgenesis [24, 25]. Sall1 is a nuclear factor that is required for maintaining the stemness of renal progenitor cells by restricting their differentiation [26, 27]. It is expressed not only in nephron progenitors but also in stromal progenitors [28]. Furthermore, *SALL1* expression is consistently high in Wilms tumor [29]. Sall1 also affects the induction of neuronal and cardiogenic differentiation markers in pluripotent mouse embryonic stem cells (ESCs) [30]. SALL1 binds

NANOG directly and is required for open heterochromatin organization in ESCs [31]. Further, transcriptional regulation by Sall1 maintains microglial identity and physiological properties in the central nervous system [32].

Table 3. Multivariate analysis of factors affecting survival using Cox proportional hazards model in 98 patients with T1 and T2 stages

Variables	Disease-free survival	
	HR (95% CI)	P-value
Age		
70 and older vs. <70	1.551 (0.776-3.098)	0.214
Sex		
Male vs. Female	0.711 (0.289-1.748)	0.457
Smoking status		
Smoker vs. Non smoker	0.769 (0.091-6.494)	0.809
Alcohol exposure		
Ever vs. Never	1.405 (0.635-3.107)	0.402
Stage		
I, II, III vs. IV	1.552 (0.749-3.218)	0.237
SALL1 methylation		
Yes vs. No	2.602 (1.296-5.224)	0.007*

HR: hazard ratio.

95% CI: 95% confidence interval.

* P < 0.05.

Three additional *SALL* genes have been identified to date: *SALL2* on chromosome 14q11.2, *SALL3* on chromosome 18q23, and *SALL4* on chromosome 20q11.2 [33]. SALL2 binds the neurotrophin receptor and regulates neuronal development [34]. *SALL2* promoter methylation has been reported in ovarian cancer and esophageal cancer cells [35, 36]. Aberrant hypermethylation of *SALL3* along with HPV infection was found to be related to carcinogenesis in cervical cancer [9]. Moreover, *SALL3* was found to be silenced by DNA methylation and this protein interacts with DNA methyltransferases 3 alpha (*DNMT3A*) in hepatocellular carcinoma [37]. Another report showed that *SALL3* hypermethylation reduced the levels of *SALL3* mRNA in hepatocellular carcinoma [38]. Moreover, *Sall3* is an epigenetic hotspot for aberrant DNA methylation associated with abnormal placental development of cloned mice [39]. The last *SALL* gene, *Sall4*, is important for maintaining a pluripotent state in mouse embryonic stem cells, [40-42] and hypomethylation of its promoter is a common event in myelodysplastic syndrome [42]. *SALL4* is highly expressed in hepatocellular carcinoma with poor prognosis, and is often associated with chronic hepatitis B virus infection [43] and several cancers with worse prognosis [44]. SALL4 can also act as a proto-oncogene by regulating the PTEN signaling pathway in glioma cells and myeloid leukemia [45, 46].

SALL1 acts as a transcriptional repressor in

mammalian cells when fused to a heterologous DNA-binding domain [47]. *SALL1* expression is also correlated with the expression of *CDH1*, and this is consistent with its tumor suppressive function and suggests its potential involvement in epithelial-to-mesenchymal transition in breast cancer [7]. *SALL1* hypermethylation has been described in several malignancies including non-small cell lung cancer [8], prostate cancer [8], acute lymphocytic leukemia [48], chronic lymphocytic leukemia [49], and colon cancer [8]. The frequency of *SALL1* promoter methylation was determined to be 4/16 (25.0%) in non-small cell lung cancer, 5/14 (35.7%) in prostate cancer, 69/78 (88.5%) in chronic lymphocytic leukemia, and 44/53 (83.0%) in colon cancer [8, 49]. Both the inactivation of *SALL1* through methylation and the loss of its transcriptional repressor function in different cancers are supportive of its role in tumor progression and metastasis.

Early reports from our group noted that the expression of *SALL3* mRNA is lost in HNSCC as a consequence of DNA methylation. *SALL3* methylation was also found in 64.8% of primary tumor specimens [15]. A concurrent analysis showed that the methylation of *SALL1* is significantly correlated with *SALL3* methylation. Furthermore, hypermethylation of both genes was associated with significantly shorter DFS, compared to that without methylation of both genes. Additionally, *CDH1* promoter methylation has been detected in many tumor types [50]. Low *CDH1* expression is associated with an increased risk of late cervical lymph node metastasis in stage I and II oral cancer patients [51], and downregulation of *CDH1* predicts delayed neck metastasis in stage I and II oral carcinoma after partial glossectomy [52]. We found that aberrant patterns of promoter methylation in primary tumors are indicators of increased risk of recurrence in patients with early stage HNSCC.

Thus, detecting aberrant *SALL1* methylation can serve as a means to identify patients at high risk for relapse. We demonstrated for the first time that *SALL1* mRNA is downregulated in HNSCC due to DNA methylation; this might be a discriminative event for early T1/T2 HNSCC progression that is associated with DFS. Our findings suggest that these methylation markers can be used clinically to identify patients that could benefit from adjuvant therapy after initial surgical treatment; however, this must be confirmed in additional prospective studies using other HNSCC patient groups.

Supplementary Material

Supplementary figures and tables.
http://www.jcancer.org/v09p0941s1.pdf

Competing Interests

The authors have declared that no competing interest exists.

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