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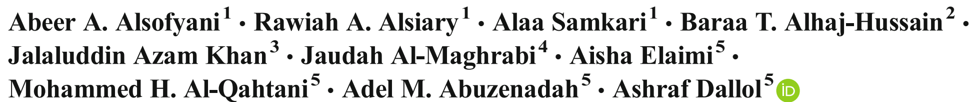
HUMAN GENETICS • ORIGINAL PAPER

Prognostic potential of *KLOTHO* and *SFRP1* promoter methylation in head and neck squamous cell carcinoma



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Abstract Hypermethylation in the CpG island promoter regions of tumor suppressors is known to play a significant role in the development of HNSCC and the detection of which can aid the classification and prognosis of HNSCC. This study aims to profile the methylation patterns in a panel of key genes including *CDKN2A, CDKN2B, KLOTHO (KL), RASSF1A, RARB, SLIT2,* and *SFRP1*, in a group of HNSCC samples from Saudi Arabia. The extent of methylation in these genes is determined using the MethyLight assay and correlated with known clinicopathological parameters in our samples of 156 formalin-fixed and paraffin-embedded HNSCC tissues. *SLIT2* methylation had the highest frequency (64.6%), followed by *RASSF1A* (41.3%), *RARB* (40.7%), *SFRP1* (34.9), *KL* (30.7%), *CKDN2B* (29.6%), and *CKDN2A* (29.1%). *KL* and *SFRP1* methylation were more predominant in nasopharyngeal tumors (P = 0.001 and P = 0.031 respectively). Kaplan Meier analysis showed that patients with moderately differentiated tumors who display *SFRP1* methylation have significantly worse overall survival in comparison with other samples. In contrast, better clinical outcomes were seen in patients with *KL* methylation. In conclusion, our findings suggest that the detection of frequent methylation in *SFRP1* and *KL* genes' promoters could serve as prognostic biomarkers for HNSCC.

Keywords Head and neck squamous cell carcinoma · Promoter region hypermethylation · KLOTHO methylation · SFRP1 methylation · Prognostic biomarkers

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Introduction

HNSCC is characterized as a heterogeneous and molecularly complex set of cancers that are caused by a variety of genetic and epigenetic aberrations that affect important mechanisms such as signal transduction, DNA repair, apoptosis, and cell cycle regulation pathways (Scully et al. 2000; Stadler et al. 2008). DNA cytosine hypermethylation within CpG island promoter regions is a chief epigenetic modification that induces the silencing of several tumor-related genes (Jones and Baylin 2002). Aberrant DNA methylation of several tumor-related genes has been frequently found in HNSCC (Czerninski et al. 2009; Demokan 2011; Hasegawa et al. 2002; Meng et al. 2016; Rigi-Ladiz et al. 2011). Many studies show that epigenetic aberrations in the promoter area of tumor suppressor genes play an important role in the initiation and progression of HNSCC (Cai et al. 2016; Fendri et al. 2009; Worsham et al. 2012). Hypermethylation of such genes is linked with clinical outcomes such as tumor stage, tumor size, and lymph node metastasis, and worse outcomes in HNSCC (Demokan 2011; Hasegawa et al. 2002; Ren et al. 2015).

Aberrant methylation of *RASSF1A* is associated with HNSCC stage (Hasegawa et al. 2002) and lymph node metastasis in nasophageal carcinomas (Fendri et al. 2009), as well as overall survival of oral squamous cell carcinoma (Huang et al. 2009). The down-regulation of *SFRP1* by promoter hypermethylation is linked with tumor grade and stage (Demokan 2011) and worse survival (Ren et al. 2015). Moreover, methylated *SFRP1* is associated with an elevated risk of recurrence in esophageal cancer (Liu et al. 2011). Methylated *RARB* is also found to be associated with highly differentiated nasopharyngeal tumors, tumor stage, and lymph node metastasis of nasopharyngeal carcinomas (Fendri et al. 2009). Aberrant methylation of the *CDKN2B* gene is associated with early stages of carcinogenesis in HNSCC (Xie et al. 2013). *CDKN2A* methylation is also associated with poorly differentiated tumor (Steinmann et al. 2009), lymph node metastasis, tumor stage, and size in HNSCC (Hasegawa et al. 2002). *SLIT2* promoter methylation is associated with poor patient outcome (Maiti et al. 2015).

The full extent of DNA methylation patterns in HNSCC carcinogenesis is yet to be elucidated. Understanding such patterns and the subsequent pathways affected will improve our understanding of HNSCC pathogenesis and may eventually lead to better clinical management. Current clinical management of HNSCC needs improvement as such cancers have been associated with worse prognosis compared to other types of tumors (Economopoulou et al. 2016; Esteller 2002). Overall, the five-year survival rate for HNSCC patients for all sites and stages is <50%, with significantly lowered post-treatment quality of life (Carvalho et al. 2005; Worsham et al. 2012).

Promoter methylation events of the *CDKN2A* (Cyclin-dependent kinase inhibitor-2A), *CDKN2B* (Cyclin-dependent kinase inhibitor-2B), *KL (KLOTHO)*, *RASSF1A* (Ras association (RalGDS/AF-6) domain family member 1A), *RARB* (Retinoic acid receptor beta), *SLIT2* (slit guidance ligand 2), and *SFRP1* (soluble frizzled receptor protein-1) genes have been commonly implicated in different tumorigenesis pathways. In the present study, we aim to assess the aberrant promoter methylation of these genes in HNSCC patients from Saudi Arabia and examine their potential as biomarkers for the disease.

Material and methods

Patients' samples

This retrospective study includes 156 formalin-fixed Paraffin-embedded (FFPE) blocks containing tissue that met the World Health Organization (WHO) criteria for squamous cell carcinoma of the head and neck. Tissues were retrieved from the department of pathology, King Abdulaziz University Hospital, and from King Abdulaziz Medical City, National Guard Health Affairs, Jeddah, Saudi Arabia, covering the period 2005 through 2014. The samples consisted primarily of tumors from nasopharyngeal tumors (n = 52, 33%), larynx (n = 20, 13%), and other oral cavity sites [tongue (n = 53, 34%), buccal site (n = 14, 9%), lip (n = 13, 8%), and undefined (n = 4, 3%)]. The relevant clinicopathological data (Table 1) (gender, age, grade, lymph node status (LN), and tumor size (T)) were obtained from the patients' records after obtaining the relevant ethical approvals (Unit of Biomedical Ethics, King Abdulaziz University Hospital No. 1127–13 and King Abdulaziz Medical City No. RJ13/042/J). Genomic DNA was

Table 1 Clinicopathological characteristics of the HNSCC cohort used in this study

	N	%
Gender		
Female	57	37
Male	99	63
Age at diagnosis (years)		
Mean ± SD	52.6 ± 16.2	
Range	(11–96)	
Younger age < 45	53	34
Older age > 45	103	66
Anatomical location		
Oral cavity		
Lip	13	8
Tongue	53	34
Buccal	14	9
Others	4	3
Larynx	20	13
Nasopharyngeal	52	33
Histological grade		
Grade 1 (well differentiated)	43	28
Grade 2 (moderately differentiated)	60	38
Grade 3 (poor differentiated)	19	12
Grade 4 (undifferentiated)	20	13
Unknown	14	9
Lymph node status		
LN+	54	35
LN-	88	56
Unknown	14	9
Tumor size		
T1	88	56
T2	37	24
T3	15	10
T4a	2	1
Unknown	14	9
Alive	106	68
Dead	50	32

extracted from the FFPE archival tissues utilizing the Qiagen QIAMP FFPE Tissue DNA extraction kit, following the manufacturer's protocol.

Sodium bisulfite modification and MethyLight assay

Up to 500 nanograms of extracted DNA was incubated with sodium bisulfite to convert unmethylated cytosine residues to uracil, leaving methylated cytosine unchanged, utilizing Qiagen Epitect Bisulfite Conversion Kit. The methylation pattern of the promoter regions of seven candidate genes (*CDKN2A*, *CDKN2B*, *KL*, *RASSF1A*, *RARB, SLIT2*, and *SFRP1)* was assessed using the MethyLight assay that is specific to bisulfite modified DNA, utilizing the primers and probes sequences previously described (Dallol et al. 2011; Dallol et al. 2012; Dallol et al. 2015; Feng et al. 2008; Kuroki et al. 2003). *COL2A1* was used as reference gene to normalize the methylation value of the input DNA for the targeted gene to the total amount of input DNA. In order to verify the specificity of the Methylight assay, a fully methylated DNA that had been treated with *Sss*I-methyltransferase (Qiagen) was used as a positive control and fully unmethylated DNA as a negative control (Qiagen), by which methylated and unmethylated alleles were discriminated.

The percentage of fully methylated reference (PMR) was calculated by dividing the 1) ratio of the quantity of targeted gene to *COL2A1* in a sample over 2) the ratio of the targeted gene to *COL2A1* in the *M.SssI*-methylated and bisulfite-treated control, and then 3) multiplying by 100. Samples where the PMR value was above or equal to 10% were considered positive for methylation, whereas samples with a PMR value below 10% were considered unmethylated.

Statistical analysis

All data analysis was carried out using SPSS version 24 (IBM Crop., N.Y., USA). Fischer's exact test (two-sided) was applied to evaluate the significance of the associations between methylation events and clinical parameters. Univariate and multivariate logistic-regression models were used to study the association between methylation level and clinical variants. The results of all models are reported using the odds ratio (OR), regression coefficient (B), and *P* value. The Kaplan-Meier test was used to estimate the impact of methylation patterns on overall survival. Overall survival was defined as the time (in months) from the date of diagnosis to the last recorded date of being alive or death. Two-sided *P* values less than 0.05 were considered significant.

Results

We screened 156 HNSCC patients elected on the basis of the availability of tissue block and patient data. The tumor tissue samples includes tissues that are surgically-resected from 57 females (36.5%) and 99 males (63.5%). The average age at diagnosis was 53 years (range, 11–96 years); 103 (66%) patients were under 45 years and 53 (34%) patients were above 45. Patient and tumor characteristics are detailed in Table 1. The methylation levels of the seven-gene panel used in this study was first confirmed to be tumor-specific by comparative analysis of matched non-tumorous tissues (Fig. 1). *SLIT2* methylation showed the highest frequency in HNSCC tumor tissues ($n = 122$, 81%), followed by *RASSF1A* ($n = 78$, 52%), *RARB* ($n = 77$, 50%), *CKDN2A* ($n = 55$, 48%), *SFRP1* ($n = 66$,

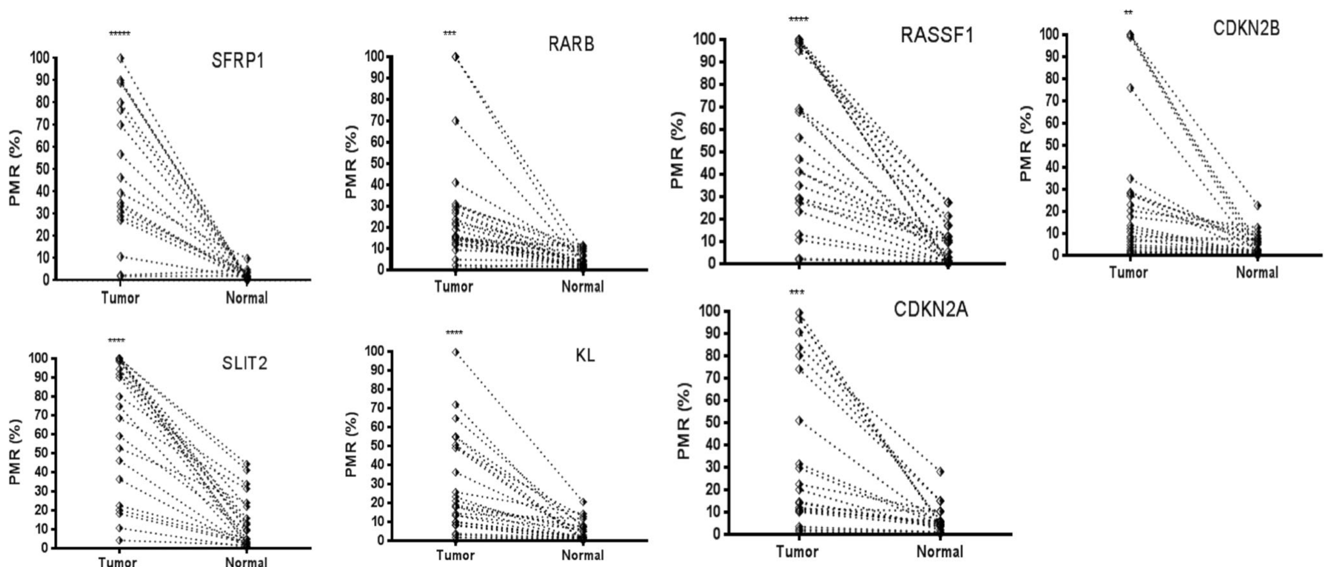

Fig. 1 Tumor-specific methylation identified for the genes analyzed in this study. % PMR is percentage of methylated ratio

47%), *KL* ($n = 58$, 40%), and *CKDN2B* ($n = 56$, 36%). Only four cases (2.5%) exhibited significant methylation in all seven genes; interestingly, these cases were histologically classified as high grade tumors (grade 3). In contrast, three cases (2%) did not exhibit any detectable methylation for any of the seven genes and these cases were histologically classified as low grade tumors (grade 1) category. *SFRP1* methylation was significantly associated with the hypermethylation of *CDKN2A, KL,* and *SLIT2* ($p < 0.001$, $p = 0.022$, and $p = 0.050$, respectively). However, *RARB* methylation was negatively associated with *CDKN2A* ($p = 0.046$) and positively associated with *KL* ($p = 0.045$). Figure 2 illustrates the methylation frequency of the candidate genes in different tumor locations (i.e., nasopharyngeal, larynx, and oral cavity).

A multivariate logistic regression model was applied to assess the potential confounding effects of clinical variables on promoter methylation events for the seven genes in different settings. Adjusted models show that tumors originated from larynx were significantly associated with methylation events in both *CDKN2A* ($p = 0.045$) and *CDKN2B* ($p = 0.009$). In addition, tongue tumors exhibited significant levels of *CDKN2B* methylation ($p = 0.012$). Moreover, nasopharyngeal tumors were significantly associated with methylated *CDKN2B* ($p = 0.016$) and methylated *SFRP1* ($p = 0.018$). Furthermore, methylation of both *CDKN2A* and *SLIT2* promoters were weakly associated with moderately differentiated (grade 2) tumors originating from the larynx site ($p = 0.039$).

In terms of association with age, younger patients (≤ 45 years old) demonstrated more frequent, albeit not statistically significant, *SFRP1* methylation compared to older patients ($p = 0.053$). Whereas, other methylated genes showed no significant association with age. In term of lymph node involvement, there was no significant correlation identified with the methylation of any gene. However, there was a significant association between tumor location and the methylation levels of *CDKN2A, KL, SFRP,* and *SLIT2* ($p = 0.001$, $p = 0.004$ $p = 0.00$, and $p = 0.001$ respectively). Moreover, undifferentiated tumors (i.e., high grade tumors, grade 3) exhibited a significant association with methylation events for both *KL* ($p = 0.002$) and *SFRP1* ($p = 0.001$).

Depending on methylation status, the data were subgrouped by tumor location, gender, tumor grade, and age. We found that *SFRP1* and *CDKN2B* methylation were more frequent in young nasopharyngeal cancer male patients (≤ 45 years old; $p = 0.001$ and $p = 0.024$, respectively). In contrast, *KL* promoter methylation was a frequent event in young female nasopharyngeal cancer patients ($p = 0.033$). Male patients with tumors originating from the larynx exhibit a weak correlation between moderately differentiated tumors and methylation events of *SLIT2* ($p = 0.039$), *CDKN2A* ($p = 0.006$), and *CDKN2B* ($p = 0.055$). Tongue cancers from male patients were associated with low methylation levels of *SLIT2* ($p = 0.031$) in poorly differentiated tumors and low methylation levels of *CDKN2A* in moderately or well-

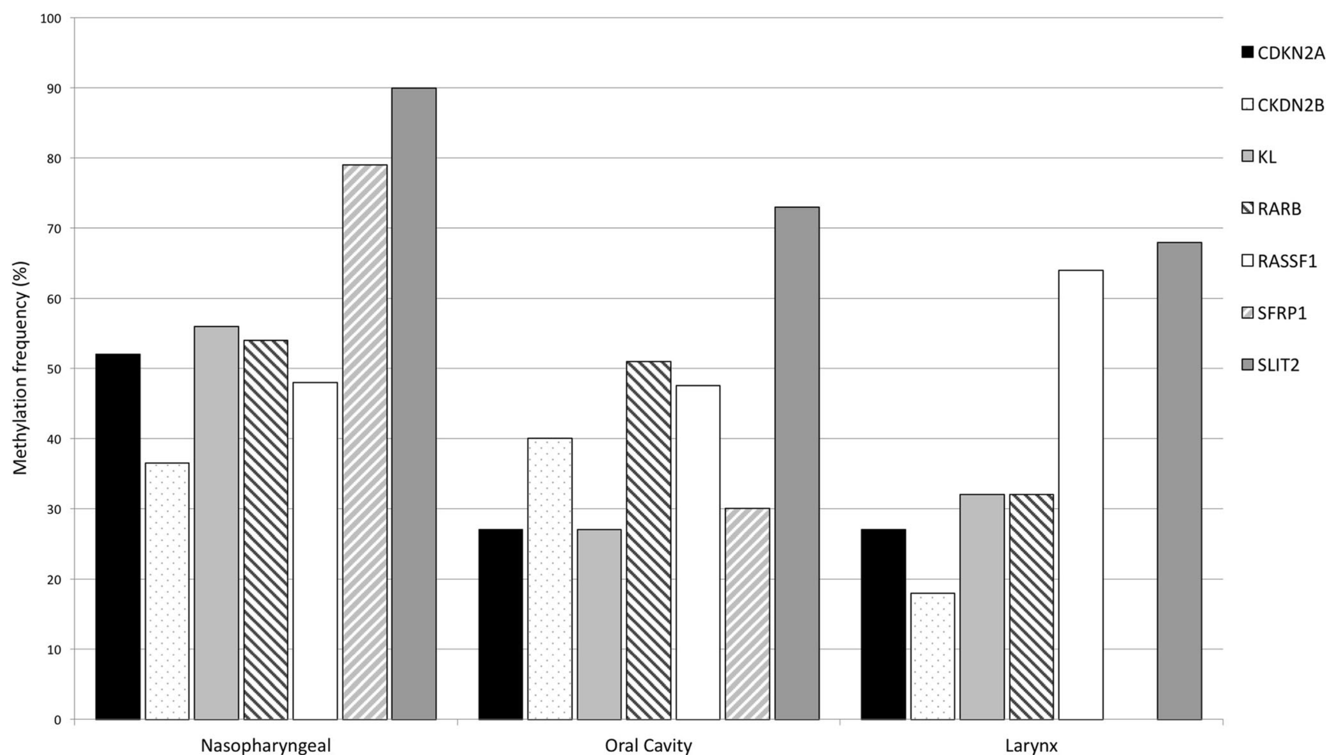

Fig. 2 Methylation frequency as determined in the HNSCC tissues indicated in x-axis

differentiated tumors ($p = 0.025$ and $p = 0.027$, respectively). No significant association between methylation patterns and overall survival for the seven genes were identified, as confirmed by Kaplan-Meier survival analysis. However, when stratified according to tumors grade, a significant association between *SFRP1* methylation and poor survival in patients with moderately differentiated tumors (grade 2) was observed ($p = 0.003$). On the other hand, *KL* methylation correlated with favorable prognosis ($p = 0.010$) in nasopharyngeal cancer patients (Fig. 3).

Discussion

Epigenetic aberrations of several tumor-related genes have been reported recently as one of the common inactivation mechanisms in head and neck cancers (Meng et al. 2016). In this study, we analyzed DNA hypermethylation of seven genes in 156 cases of HNSCC. Methylation of six genes (*CDKN2A, CDKN2B, RASSF1A, RARB, SLIT2*, and *SFRP1*) was previously reported in head and neck cancers (Fendri et al. 2009; Gröbe et al. 2013; Maiti et al. 2015; Marsit et al. 2006; Maruya et al. 2004). Frequent hypermethylation of *KL* has been recently reported in breast and cervical cancer (Dallol et al. 2015; Lee et al. 2010). To our knowledge, this is the first study to show a significant association between the presence of methylation of the *KL* promoter and young female with nasopharyngeal cancer patients (≤ 45 years old; $p = 0.033$). It is interesting to hypothesize that *KL* hypermethylation may be connected with the female physiology and thus more frequent in female cancer patients (Dallol et al. 2015; Rubinek et al. 2012). Wolf et al. (2008) reported an association between women with breast cancer with *BRCA1* mutations and lower *KL* expression (Wolf et al. 2008). Lee et al. (2010) reported that epigenetic silencing of *KL* was associated with aberrant activation of the Wnt pathway in invasive cervical carcinoma (Lee et al. 2010). *KL* is a hormone that plays important roles in longevity and cellular homeostasis (Dallol et al. 2015; Kurosu et al. 2005). Moreover, several studies have reported that *KL* displays a tumor suppressive role in many cancers by inhibiting the growth factor receptor signaling (IGF-1R signaling) pathway that stimulate cell proliferation and migration (Lee et al. 2010). Taken together, these data and the literature suggest that *KL* methylation is a potential tumor suppressor gene for HNSCC progression.

Previously, it was reported that inactivation of *SLIT2* by methylation had prognostic value in HNSCC as it was one of the early events in the development of neoplasm in head and neck (Maiti et al. 2015). In the current study, *SLIT2* was the most frequently methylated gene, occurring in about ($n = 122$, 80.8%) of all cases, which suggest that *SLIT2* methylation status might be an early event in HNSCC tumorigenesis. The *SLIT2* gene encodes a small non-coding RNA called microRNA-218 (miRNA-218) within one of its introns (Zhang et al. 2013). It has been shown that miRNA-218 functions as a tumor suppressor that inhibits the expression of oncogenes (Kinoshita et al. 2012). miRNA-218 specifically targets the pro-migration and invasion candidate oncogene *LAMB3* in HNSCC cells (Kinoshita et al. 2012). MicroRNA-218 is epigenetically regulated by *SLIT2* gene as miRNA-218 was shown recently to be suppressed by *SLIT2* hypermethylation in oral SCC (Rodriguez et al. 2004). Therefore, aberrant promoter methylation of *SLIT2* is likely to lead to transcriptional silencing of miRNA-218 in several cancer tissues, thus reducing its tumor suppressor activities.

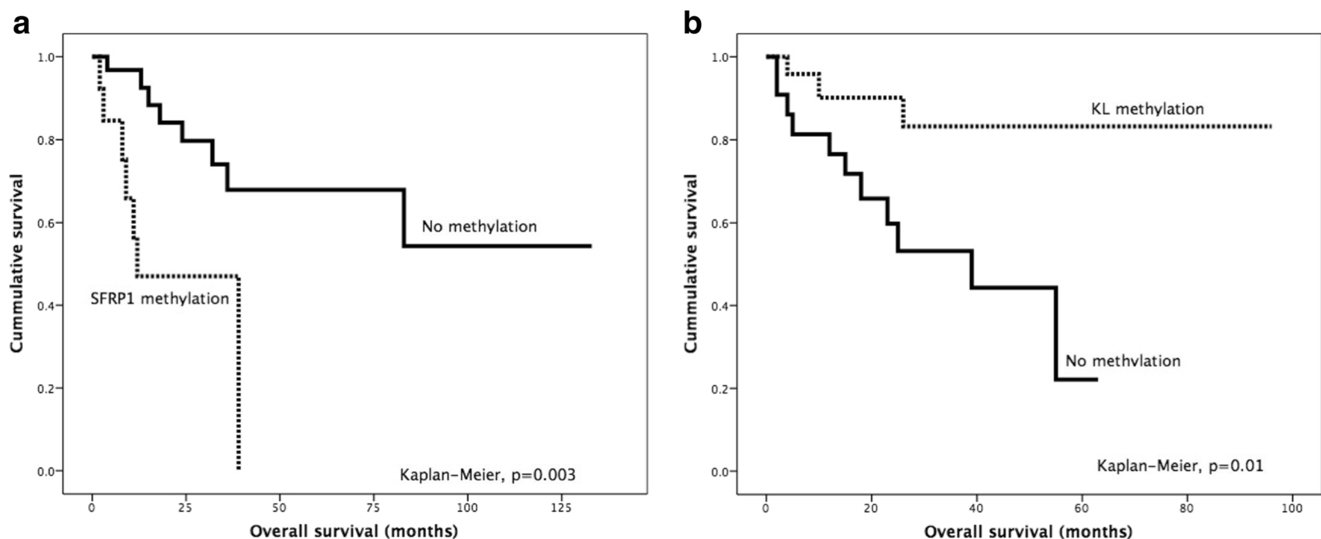

Fig. 3 Kaplan-Meier survival analysis demonstrating the effect of *SFRP1* (a) or *KL* (b) methylation on overall survival

In line with other studies, *CDKN2A, CDKN2B, RARB*, and *RASSF1A* were frequently methylated in our samples. However, our results failed to find a significant correlation between such genes and age, gender, tumor size, and lymph node involvement. This could indicate a different underlying mechanism(s) of HNSCC carcinogenesis in our samples.

This study also showed that a number (n = 66, 46.8%) of patients with HNSCC exhibited significant hypermethylation in the *SFRP1* promoter. This finding is lower than the percentage of *SFRP1* promoter hypermethylation reported in HNSCC previously (Marsit et al. 2006). However, this study reports that *SFRP1* methylation is more frequent in young male nasopharyngeal cancer patients (n = 15, 29%), (p = 0.001). Abnormal methylation of *SFRP1* is likely to result in an aberrant Wnt/β-catenin signaling pathway, which is required for cell differentiation, proliferation, and adhesion (Ren et al. 2015). Compared with other head and neck tumors, early distant metastasis and high rates of local invasion have been observed in patients with nasopharyngeal cancer (Kinoshita et al. 2012; Lee et al. 1992). Our finding shows the close association of *SFRP1* methylation with clinical outcomes in HNSCC patients. Kaplan–Meier analysis showed that patients with moderately differentiated tumors who display *SFRP1* methylation have significantly worse overall survival. A similar finding between *SFRP1* promoter methylation and unfavorable prognosis has been demonstrated for brain tumors (Majchrzak-Celińska et al. 2016), bladder cancers (Marsit et al. 2005), and breast cancer (Veeck et al. 2006). Conversely, a better clinical outcome was seen in patients with *KL* methylation, unlike the previous report by Xie et al., whose study linked *KL* methylation with poor prognosis in hepatocellular carcinoma (Xie et al. 2013).

Our findings show that by examining the methylation frequency of the seven genes, HNSCC tumors can be stratified accordingly. Therefore, the methylome of this group of tumors is likely to reveal interesting biomarkers that can help provide a better understanding of this disease and its progression.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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