High SEPT9_v1 Expression Is Associated with Poor Clinical Outcomes in Head and Neck Squamous Cell Carcinoma


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Abstract

The purpose of this work was to determine SEPT9_v1 expression levels in head and neck squamous cell carcinoma (HNSCC) and to analyze whether SEPT9_v1 expression is relevant to clinical outcomes. Recently, the SEPT9 isoform SEPT9_v1 has been implicated in oncogenesis, and methylation of the SEPT9 promoter region was reported in HNSCC. These findings led us to hypothesize that SEPT9_v1 could be differently expressed in HNSCC. To determine whether SEPT9_v1 is expressed in HNSCC, tissue microarray immunohistochemical analysis was performed using a SEPT9_v1-specific antibody. Tissue microarrays stained with a polyclonal SEPT9_v1-specific antibody was used to determine protein expression levels in HNSCC tissue samples, some with known clinical outcomes. This analysis showed that SEPT9_v1 is in fact highly expressed in HNSCC compared with normal epithelium, and high expression levels directly correlated with poor clinical outcomes. Specifically, a high SEPT9_v1 expression was associated with decreased disease-specific survival (P = .012), time to indication of surgery at primary site (P = .008), response to induction chemotherapy (P = .0002), and response to chemotherapy (P = .02), as well as advanced tumor stage (P = .012) and N stage (P = .0014). The expression of SEPT9_v1 was also strongly correlated with smoking status (P = .00094). SEPT9_v1 is highly expressed in HNSCC, and a high expression of SEPT9_v1 is associated with poor clinical outcomes. These data indicate that SEPT9_v1 warrants additional investigation as a potential biomarker for HNSCC.

Introduction

Head and neck cancers are a complex heterogeneous set of solid tumor malignancies marked by diverse molecular mechanisms. Alterations in p53 [1–5], loss of p16 [3,6–9], amplification of cyclin D1 [3,4,6,10], and overexpression of epidermal growth factor receptor [3,11–13] are all important mechanisms in the progression of head and neck cancers. Recently, methylation of the SEPT9 promoter was identified in a genome-wide screen of head and neck cancers [14]. Methylation of the promoter region of a gene generally results in silencing of the locus.
This is achieved by condensing the chromatin which limits the transcription machinery’s access to the locus. Although this study found that SEPT9 expression was decreased in a subset of head and neck squamous cell carcinoma (HNSCC) samples studied, it did not look at each individual SEPT9 variant encoded at the locus to determine which specific variants were silenced. Because high SEPT9_v1 expression has been implicated in breast, ovarian, and prostate cancers [15–17], we were interested in determining whether SEPT9_v1 expression plays a role in HNSCC, hypothesizing that this unique variant may be upregulated whereas other variants are silenced as previously suggested in breast cancer [17].

SEPT9 belongs to a highly conserved family of proteins called Septins originally described in Saccharomyces cerevisiae. This family of proteins has proven to be important for faithful cell cycle progression and bud morphology. Mutation analysis of SEPT9 found that it functions in cell division and cell cycle progression through its inhibition of cyclin-dependent kinases necessary for progression through G2-M [18]. Because of its role in cell division and cell cycle progression, SEPT9 has been implicated in oncogenesis. SEPT9 has been shown to go through alternative splicing at both ends, resulting in a variety of transcripts, which are found to be differentially expressed. In addition, SEPT9 variants possess unique promoter sequences, allowing for distinct regulation of each variant. It seems as though altered expression of these variants is responsible for the development of malignancies because no specific mutations in either the introns or the exons of SEPT9 have been found [19].

One SEPT9 variant, SEPT9_v1, has been shown to be highly expressed in breast, ovarian, and prostate cancers [15–17]. In prostate cancer models, SEPT9_v1 interacts with hypoxia-inducible factor 1α, which increases angiogenesis [15]. In immortalized human mammary epithelial cells (IHMECs), overexpression of SEPT9_v1 results in oncogenic phenotypes including an epithelial-to-mesenchymal transition, increased cellular proliferation, invasiveness, aneuploidy, disrupted tubulin filaments, promoted binucleated cells, and abnormal localization of SEPT9_v1 to the nucleus [17]. In addition, SEPT9_v1 acts to stabilize c-Jun N-terminal kinase (JNK) and prevents its degradation [20]. The JNK signaling pathway is known to play a role in cell proliferation and tumorigenesis. Interestingly, JNK expression and subsequent kinase activity are increased in HNSCC [21].

Head and neck cancers are a heterogeneous group of cancers, which are marked by their aggressiveness and invasiveness. HNSCCs are associated with poor prognosis and clinical outcome owing to this feature, making the identification of prognostic marker(s) all the more important. On the basis of the data described here, SEPT9_v1 has the potential to emerge as an important prognostic and therapeutic biomarker for HNSCC.

**Materials and Methods**

**Tissue Microarray Construction**

Tissue microarray (TMA) 74 was constructed using HNSCC samples from 11 patients. These 11 patients provided multiple tumor samples. In five patients, normal samples were also present and analyzed as a matched set.

TMA 96 was constructed as previously described [3]. Briefly, samples were obtained from a clinical trial of 50 patients with either stage III or IV squamous cell carcinoma of the oropharynx. All samples were obtained pretreatment, and patients were subsequently followed through treatment protocols.

![Figure 1](image_url)

**Figure 1.** Composite showing representative staining in TMA 74. Panel #1 shows normal epithelium as indicated by the arrows and no SEPT9_v1 staining. Panel #2 shows low SEPT9_v1 staining. Panel #3 shows medium SEPT9_v1 staining. Panel #4 shows high SEPT9_v1 staining and perineural invasion, indicated by the arrowheads.
Immunohistochemistry

A custom rabbit polyclonal SEPT9_v1-specific antibody was raised to 17 of the unique 25 amino acids at the N-terminus of SEPT9_v1 (KKSYSGGTRTSSGRLRR) (BioCarta, San Diego, CA) [17]. Specificity of this antibody was demonstrated in previous studies [17, 20].

Immunohistochemistry was performed at the University of Michigan Histology and Immunohistochemistry Services Core Laboratory. Briefly, antigen retrieval was performed using citrate, pH 6.0, and then micro-waving for 10 minutes. Subsequent staining was performed on an auto-stainer (DAKO, Carpinteria, CA) at room temperature. Primary SEPT9_v1 or immunoglobulin class G control antibody was used at a dilution of 1:200 and incubated for 30 minutes. Anti-rabbit EnVision+ was used to detect the primary antibody. After this, the slide was incubated with DAB and counterstained with hematoxylin.

TMA Scoring

Slides were reviewed for SEPT9_v1 staining by two investigators who were blinded to clinical data (E.M. Petty and L. Stanbery). Slides were scored by a head and neck pathologist (N.J. D’Silva) who was blinded to clinical outcomes of the patients. Two criteria were used to score the slides: intensity of antibody staining and percentage of staining in the entire sample. Intensity of staining was scored on a scale of 1 to 4: 1, no or undetectable staining; 2, low staining; 3, medium staining; and 4 high staining. Samples that had insufficient tissue present were not scored or used for data analysis.

Statistics

TMA 74. Unpaired and paired t tests were used to determine P value, with P ≤ .05 being statistically relevant.

TMA 96. Per protocol, patients received one cycle of induction chemotherapy. Response to induction chemotherapy was recorded as complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD). Subjects who responded to induction chemotherapy (CR and PR) were treated with chemoradiation. Response to chemoradiation therapy was recorded as complete response and otherwise. Subjects who were SD and PD to induction chemotherapy or those who did not respond to chemoradiation received surgery with therapeutic intend. The outcomes of interest, other than

Figure 2. Probability of survival over time according to SEPT9_v1 expression. Subjects were censored (black vertical bar) when they did not experience an event.
response to induction chemotherapy and response to chemoradiation therapy, were overall survival, disease-specific survival, time to indication of surgery at primary site, and time to recurrence or second primary. For time to indication of surgery at primary site, the events were SD or PD after induction chemotherapy that required surgery at the primary site, local recurrence after chemoradiation therapy that required surgery at the primary site, and never disease-free. For the outcome of time to recurrence or second primary, the events included recurrences, relevant second primaries, and never disease-free. The covariates of interest were T stage and N stage, which were analyzed as ordinal data.

The Spearman correlation coefficient was used to evaluate associations between marker levels and ordinal variables of interest, such as induction chemoresponse and chemoradiation response T and N stage. The Cox proportional hazards model was used to relate time-to-event outcomes to marker levels and demographic and clinical variables.

All statistical analyses were done using Statistical Analysis System version 9.0 (SAS, Cary, NC). Two-tailed $P \leq .05$ was considered to be statistically significant.

**Results and Discussion**

High expression of SEPT9_v1 has been associated with the pathogenesis of several cancers and acquisition of oncogenic phenotypes in IHMECs [17]. We wanted to analyze the role of SEPT9_v1 expression in HNSCC. To this end, we used a tissue microarray constructed from patient samples and stained using a SEPT9_v1-specific antibody. Each sample was then scored by a pathologist for expression level of SEPT9_v1 (Figure 1) as well as percent staining of the total sample.

TMA 74 (Figure 1) was used to determine whether there was any correlation between SEPT9_v1 expression and normal versus tumor tissue. We determined that SEPT9_v1 was expressed highly in tumor samples versus normal samples ($P = .0033$). In addition, when comparing matched samples from the five patients studied, we saw a strong correlation between high SEPT9_v1 expression and tumor samples ($P = .0064$). This indicates that SEPT9_v1 was not originally highly expressed in the normal tissues of these patients.

Using TMA 96, we found that high expression of SEPT9_v1 was associated with several less favorable outcomes, including decreased disease-specific survival ($P = .012$; Figure 2) and time to indication...

**Figure 3.** Probability of a patient needing surgery at the primary site over time. Subjects were censored (black vertical bar) when they did not experience an event, that is, death from an unrelated event.
of surgery at primary site ($P = .008; \text{Figure 3})$. Patients with tumors with no SEPT9_v1 had virtually no need for surgery at the primary site. The indication for surgery increased, with increasing levels of SEPT9_v1 being present. SEPT9_v1 expression also correlated with the probability of disease-specific survival. Patients with no SEPT9_v1 expression had a significantly higher survival rate, whereas increasing

**Figure 4.** (a) Patient response to induction chemotherapy as a function of mean SEPT9_v1 intensity. Subjects were censored when no event was experienced. Horizontal bar, median; box, range. (b) Response to chemotherapy on the basis of SEPT9_v1 expression. Patients were classified into two groups: whether they responded to chemotherapy or all others. Horizontal bar, median; box, range; hollow circle, outlier.

**Figure 5.** T stage correlation with SEPT9_v1 intensity. Patients were grouped on the basis of tumor size. Horizontal bar, median; box, range; hollow circle, outliers.

**Figure 6.** N stage correlation with SEPT9_v1 intensity. Patients were grouped on the basis of metastasis into the surrounding lymph nodes. Horizontal bar, median; box, range; hollow circle, outliers.
levels of SEPT9_v1 resulted in decreased survival. These results indicate that high levels of SEPT9_v1 are associated with less favorable outcomes.

Patients with tumors with high SEPT9_v1 expression showed poor response to induction chemotherapy \( (P = .0002;) \) and had a higher rate of progressive disease, whereas patients with a low SEPT9_v1 expression exhibited SD. Also, patients having tumors with low SEPT9_v1 expression respond better to chemotherapy than patients with tumors having high SEPT9_v1 expression \( (P = .02;) \). The poor response to treatments seen in patients with high SEPT9_v1 may be the cause of the poor outcomes seen in these patients.

In addition, patients with stage IV cancer have higher expression of SEPT9_v1 \( (P = .012;) \) (Figure 5). Stage IV cancers have usually metastasized and are routinely associated with poor outcomes. In addition to high SEPT9_v1 expression being correlated to T stage, correlation with N stage status is also highly significant \( (P = .0014;) \) (Figure 6). This means that invasion into the surrounding lymph nodes correlates with high SEPT9_v1 expression. Interestingly, metastatic cells must gain the ability to be motile and to invade, two characteristics that are seen in IHMECs expressing SEPT9_v1. In addition, advanced T and N stage could also be a result of poor response to induction chemotherapy and chemotherapy allowing the tumors to become more advanced.

High SEPT9_v1 expression was also correlated with smoking status \( (P = .0009;) \) (Figure 7). Current smokers had high SEPT9_v1 expression. Interestingly, Bennett et al. \( [23]\) also found that SEPT9 promoter methylation correlated with smoking status. We saw no correlation with SEPT9_v1 expression and age or gender.

Although recent findings from Bennett et al. \( [14]\) showed that the SEPT9 promoter in HNSCC is methylated and protein expression is decreased, their study did not look at individual SEPT9 transcripts. Their subsequent study also showed a correlation between promoter methylation and smoking status \( [23]\). There is evidence that each transcript possesses its own promoter; therefore, methylation of one promoter may only silence one transcript, whereas up-regulation of another may occur. We have also seen different SEPT9 isoforms acting differently in breast cancer \( [17]\). For example, SEPT9_v1 and SEPT9_v3, which differ in only the first 25 amino acids, have vastly different phenotypes when expressed. Whereas SEPT9_v1 has a tumorigenic effect, SEPT9_v3 has a tumor-suppressive-like effect \( [18]\). SEPT9 also forms homogenous and heterogenous complexes with other septins to form filaments \( [19,24]\). One hypothesis is a feedback loop, where low levels of one variant triggers another variant to be expressed.

**Conclusions**

Collectively, these data suggest that a high expression of SEPT9_v1 is associated with poor clinical outcomes in HNSCC. Furthermore, we found that a high expression of SEPT9_v1 correlated with both advanced T and N stage, indicating that SEPT9_v1 may play a role in motility and invasion. This hypothesis is supported by studies of SEPT9_v1 expression in mammalian epithelial cells where increased expression of SEPT9_v1 led to increased motility and invasion. Further research needs to be conducted to determine the cause of high SEPT9_v1 expression in HNSCC, and what role SEPT9_v1 is playing in oncogenesis.

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**References**


