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ORIGINAL ARTICLE

# Hypermethylation of *RUNX3* but not *WIF1* gene and its association with stage and nodal status of tongue cancers

**G Supic[1], R Kozomara[2], N Jovic[2], K Zeljic[1], Z Magic[1]**

[1]*Institute for Medical Research, Military Medical Academy, Belgrade, Serbia;* [2]*Clinic for Maxillofacial Surgery, Military Medical Academy, Belgrade, Serbia*

**OBJECTIVES: Recent studies indicate various molecular abnormalities in oral squamous cell carcinomas (*OSCC*), including DNA methylation of tumor-associated genes. Although promoter hypermethylation of *Wnt* pathway antagonists *RUNX3* (*Runt-related transcription factor 3*) and *Wnt inhibitory factor 1* (*WIF1*) has been identified as a common event in a number of carcinomas, methylation status and the role of *RUNX3* as a possible tumor suppressor in oral and head and neck cancer are yet controversial. The aim of our study is to determine the occurrence of *RUNX3* and *WIF1* genes hypermethylation and correlation with tumor and host-related factors and prognosis in tongue carcinomas.**

**MATERIAL AND METHODS: In 76 patients with tongue carcinoma, *RUNX3* and *WIF1* genes promoter hypermethylation analysis was assessed by nested methylation-specific PCR method.**

**RESULTS: Hypermethylation of *WIF1* and *RUNX3* genes promoters was observed in 35% and 25% of carcinomas, respectively. *RUNX3* gene promoter hypermethylation was significantly associated with lymph node involvement ($P = 0.013$) and tumor stage ($P = 0.006$), but not with the overall survival. Occurrence of *RUNX3* and *WIF1* genes comethylation was associated with nodal status ($P = 0.058$) and tumor stage ($P = 0.055$).**

**CONCLUSIONS: Our findings indicate that *RUNX3* and *WIF1* are frequently aberrantly methylated and that *RUNX3* promoter methylation could be considered as a potential prognostic marker in tongue carcinoma.**

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**Keywords:** DNA methylation; oral squamous cell carcinoma; tongue carcinoma; RUNX3; WIF1; nodal and tumor stage

## Introduction

Oral squamous cell carcinoma (*OSCC*) is the eleventh most common cancer worldwide. Despite significant efforts committed in recent years to diagnosis and treatment of *OSCC*, the incidence rates are rising and the survival rates remain poor (Scully and Bagan, 2009). Recent advances in molecular biology indicated multiple genetic and epigenetic changes that cause aberrant expression and function of proteins regulating cell signaling, growth, survival, motility, angiogenesis, and cell cycle control (Williams, 2000; Scully and Bagan, 2009). Epigenetic modifications, heritable changes in gene expression that are not coded in the DNA sequence, cause transcriptional silencing of tumor suppressor genes and malignant transformation (Herman and Baylin, 2003; Shaw, 2006). The key epigenetic mechanism, DNA methylation of CpG islands in tumor-associated genes promoters, is a frequent event in *OSCC* (Shaw, 2006; Shaw *et al*, 2007; Supic *et al*, 2009, 2011).

In recent years, *Wnt* (*wingless type*)/β-catenin signaling has been increasingly implicated in the cancer initiation and malignant transformation in a wide range of human cancers, including *OSCC* (Lo Muzio, 2001; Romana, 2010). *Wnt* proteins are a large family of secreted glycoproteins that bind to specific transmembrane receptors and activate β-catenin. In a normal cell, β-catenin localizes to the cytoplasm and is continuously degraded by phosphorylation and ubiquitination. In response to binding of the *Wnt* proteins, β-catenin is stabilized, accumulated in the cytoplasm, and translocated to the nucleus, where it activates growth-promoting oncogenes and regulates cell polarity and cytoskeletal rearrangements, invasion, metastasis, and angiogenesis (Romana, 2010).

Secreted *Wnt* antagonists, *RUNX3* (*Runt-related transcription factor 3*) and *Wnt inhibitory factor 1* (*WIF1*), are negative modulators of *Wnt* signaling, which prevent β-catenin from acting as a transcriptional activator. *RUNX3* belongs to the *RUNX* family of DNA-binding transcription factors that control cell differentiation. It has been shown that *RUNX3* forms a complex with β-catenin, preventing it from acting as a transcriptional activator and attenuates *Wnt* signaling activity in gastric cancer (Ito *et al*, 2008). *RUNX3* is a downstream target of the transforming growth factor-β

Correspondence: Gordana Supic, Institute for Medical Research, Military Medical Academy, Crnotravska 17, 11002 Belgrade, Serbia. Tel: +381 60 4022202, Fax: +381 11 2662722, E-mail: gogasupic@sezampro.rs
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(TGF-$\beta$) signaling pathway, the key pathway that influences apoptosis, angiogenesis, cell adhesion, and invasion (Subramaniam *et al*, 2009). Although it has been shown that *RUNX3* has a tumor suppressor role in a number of cancer types, including *OSCC* (Gao *et al*, 2009), a recent study revealed that *RUNX3* may also have an oncogenic role in *HNSCC* (Tsunematsu *et al*, 2009). *WIF1*, a secreted *Wnt* antagonist, is a downstream gene of the *Wnt*/$\beta$-catenin pathway, which exerts inhibition through direct binding to *Wnt* proteins (Reguart *et al*, 2004). The expression of *WIF1* is aberrant in numerous types of cancer, including *HNCSS* (Rhee *et al*, 2002). *WIF1* inhibits death receptor–mediated apoptosis and promotes the invasive growth of *HNSCC* (Yang *et al*, 2006).

As mutations of genes responsible for the *Wnt* activation, such as $\beta$-catenin and adenomatous polyposis coli (*APC*), rarely occur in oral and head and neck cancers (Iwai *et al*, 2005), alternative mechanism that could likely lead to the activation of *Wnt* pathway is silencing of the *Wnt* antagonists through promoter hypermethylation. Recent studies reveal that the expression of *RUNX3* is downregulated by hypermethylation in a number of cancer types, including oral (Gao *et al*, 2009), esophageal (Torquati *et al*, 2004), intestinal (Ito *et al*, 2008), colorectal (Ahlquist *et al*, 2008), lung (Licchesi *et al*, 2008), and breast cancer (Park *et al*, 2011). *WIF1* was found to be silenced by methylation in various human carcinomas including oral (Pannone *et al*, 2010), nasopharyngeal (Lin *et al*, 2006; Chan *et al*, 2007), esophageal (Clément *et al*, 2008), lung (Wissmann *et al*, 2003; Mazieres *et al*, 2004), breast (Ai *et al*, 2006), bladder (Urakami *et al*, 2006), and colon cancer (Lee *et al*, 2009).

The objectives of our study are to determine the occurrence of *RUNX3* and *WIF1* genes hypermethylation and correlation of methylation status with tumor and host-related factors such as tumor size, stage, nodal status, age, gender, smoking, alcohol use, and overall survival in primary squamous cell carcinoma of the tongue.

## Materials and methods

*Patients*

Seventy-six primary squamous cell carcinomas of tongue were obtained from the Clinic for Maxillofacial Surgery, Military Medical Academy, Belgrade, Serbia, after the approval from the Institutional Review Board, between 2000 and 2008. The patients were not treated with chemotherapy or radiation prior to surgery. Samples were subjected to histologic evaluation by the experienced pathologist and were frozen at −80°C until DNA extraction.

*Methylation-specific polymerase chain reaction*

DNA from tumor samples was extracted using TRIZOL reagent (Invitrogen, Karlsruhe, Germany) and subsequently modified by sodium bisulfite treatment using the EpiTec Bisulfite Kit (Qiagen, Hilden, Germany). Methylation status was assessed by multiplex nested methylation-specific PCR (MS-PCR), as previously described (Herman *et al*, 1996; Palmisano *et al*, 2000). Investigator performing the MS-PCR was blinded to patient outcomes. Bisulfite-modified DNA was initially amplified with flanking primers without preferentially amplifying methylated or unmethylated DNA. Resulting amplification products were 50-fold diluted and used as the templates for MS-PCR with primers specific to discriminate methylated or unmethylated alleles. Primer sequences of *RUNX3* and *WIF1* have previously been described (Licchesi *et al*, 2008). Lymphocyte DNA from healthy individuals was used as unmethylated control, and the same DNA methylated *in vitro* by CpG methylase *SssI* (New England Biolabs, Beverly, MA, USA) was used as methylated control. MSP products were verified by 2% agarose gel electrophoresis.

*Statistical analysis*

Data were analyzed by the SPSS 16.0 software. Study of correlation between *RUNX3* and *WIF1* genes promoter methylation and clinicopathological variables was performed using the Chi-squared test or Fisher's exact probability test. Overall survival was determined from the date of surgery until death from any cause, and Kaplan–Meier survival curves were compared by the log-rank test. Cox proportional hazards regression analysis was performed to estimate the hazard ratios (HR), with 95% confidence interval (95% CI). Variables found statistically significant in the univariate analysis, including variable with significance level below 20%, were subsequently analyzed together in multivariate analysis. The Cox model was performed using the forward stepwise method, which removed variables with $P < 0.1$. A two-sided value of $P$ values $<0.05$ was considered to be of statistical significance.

## Results

Demographic and clinical characteristics of the studied patient group with squamous cell carcinoma of tongue are presented in Table 1. Studied cohort consisted of 59 male and 17 female patients, age ranged from 39 to 77 (median age 58 years). The majority of the patients, 72% (55/76), were diagnosed with advanced tumor stages III, and 28% (21/76) of patients had confirmed tumor stage II, according to the TNM classification. Fifty-seven cases (75%) were node positive, while 25% of cases were node negative.

Hypermethylation of *WIF1* gene promoter was observed in 39% (28/76), while *RUNX3* was hypermethylated in 34% (26/76) of tongue carcinoma samples. Representative examples of methylation status analysis are presented in Figure 1.

While a significantly increased prevalence of *RUNX3* gene methylation was observed in men compared with women ($P = 0.040$, Fisher's test), there was no significant association between patient gender and methylation of *WIF1* gene, Table 2. No association of patient's age at diagnosis, smoking, or alcohol use with the

**Table 1** Clinicopathological characteristics of the studied cohort

| *Variables* | N |
|---|---|
| Sex | |
| Male | 59 |
| Female | 17 |
| Age[a] | |
| < 58 | 30 |
| ≥ 58 | 46 |
| Smoking | |
| Never | 10 |
| Ever | 66 |
| Alcohol use | |
| Low | 24 |
| Moderate | 26 |
| High | 26 |
| Stage | |
| II | 21 |
| III | 55 |
| Tumor size | |
| T1/2 | 60 |
| T3/4 | 16 |
| Nodal status | |
| N 0 | 19 |
| N + | 57 |
| Recurrence | |
| No | 34 |
| Yes | 42 |
| Histologic grade | |
| 0 | 24 |
| 1 | 38 |
| 2 | 14 |
| Nuclear grade | |
| 0 | 16 |
| 1 | 36 |
| 2 | 24 |

*N*, total number of patients.
[a]Age, according to median value of 58 years (range 36–81).

occurrence of *RUNX3* or *WIF1* gene methylation was observed in the studied cohort, Table 2.

We observed a statistically significant correlation of the presence of *RUNX3* gene promoter hypermethylation with the tumor stage (stage III *vs* II), $P = 0.006$, Fisher's test, and the lymph node involvement (N positive *vs* N negative), $P = 0.013$, Fisher's test, Table 3. Hypermethylation of *WIF1* gene was not associated with tumor stage and the presence of lymph node metastases. Hypermethylation of either of the studied genes was not associated with nuclear or histologic grade, Table 3.

Comethylation of *RUNX3* and *WIF1* genes showed a tendency toward occurrence in patients with the presence of lymph node metastases, $P = 0.058$, Fisher's test, and advanced tumor stage, $P = 0.055$, Fisher's test, Table 3.

Significant decrease in overall survival with known prognostic indicators, such as lymph node involvement, tumor stage, and tumor size, was observed in patients with tongue carcinoma, as expected. Univariate analysis revealed that tumor size (T3/4 *vs* T1/2) (HR = 1.446, 1.032–2.026, 95% CI, $P = 0.032$), nodal status (HR = 2.955, 1.163–7.508, 95% CI, $P = 0.023$), and tumor stage (HR = 2.653, 1.119–6.290, 95% CI, $P = 0.027$) were significant prognostic indicators for overall survival. Variables found statistically significant in the univariate analysis, including alcohol use (HR = 1.497, 0.921–2.432, 95% CI, $P = 0.103$) and smoking (HR = 1.507, 0.899–2.527, 95% CI, $P = 0.120$), as variables with significance level below 20%, were subsequently analyzed together in multivariate analysis. Nodal status persisted as an independent prognostic factor for the overall survival (HR = 2.955, 1.163–7.508, 95% CI, $P = 0.023$), while contribution of other variables was lost in the final multivariate model.

No association was observed between methylation of *RUNX3* or *WIF1* hypermethylation gene alone or comethylation of these genes and overall survival in patients with tongue carcinoma, Figure 2.

## Discussion

Aberrant epigenetic changes were observed during the development and progression of oral cancer in genes regulating cell signaling, growth, motility, angiogenesis, and cell cycle control, including *Wnt* pathway (Williams, 2000; Lo Muzio, 2001; Shaw, 2006; Romana, 2010). In recent years, there has been a rapid increase in potential clinical implications of epigenetic changes and DNA methylation in early diagnosis of oral cancer (Cao *et al*, 2009), as a prognostic marker for assessing the aggressiveness of the disease (Sailasree *et al*, 2008) and as a prognostic marker of survival in patients with oral carcinoma (Supic *et al*, 2009; Su *et al*, 2010). In addition, owing to the reversibility of epigenetic changes, novel epigenetic therapy has been developed, which has been promising in a number of tumor types, but yet to be explored in *OSCC* (Magic *et al*, 2009).

In the present study, we observed frequent hypermethylation of *Wnt* inhibitor *WIF1* and *RUNX3* genes and a significant correlation between *RUNX3* promoter methylation and lymph node involvement and advanced disease stage in tongue carcinoma. However, no significant association was observed between *RUNX3* or *WIF1* hypermethylation and overall survival of patients with tongue carcinoma. Recent investigations suggest

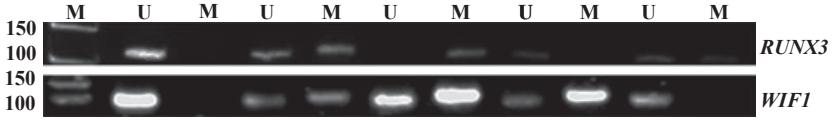


**Figure 1** Representative samples of methylation-specific PCR analysis in tumor tissue of patients with tongue carcinoma. *M*, DNA marker; M, methylated gene; U, unmethylated gene

**Table 2** Association of *RUNX3* and *Wnt inhibitory factor 1* (*WIF1*) genes promoter hypermethylation with demographic variables of patients with tongue carcinoma

| *Variables* | *RUNX3 Unmet* | *RUNX3 Met* | P[a] | *WIF1 Unmet* | *WIF1 Met* | P[a] | *Comethylation* – | *Comethylation* + | P[a] |
|---|---|---|---|---|---|---|---|---|---|
| Sex | | | | | | | | | |
| Male | 35 | 24 | 0.040 | 35 | 24 | 0.259 | 45 | 14 | 0.500 |
| Female | 15 | 2 | | 13 | 4 | | 15 | 2 | |
| Age[b] | | | | | | | | | |
| < 58 | 19 | 11 | 0.715 | 21 | 9 | 0.318 | 23 | 7 | 0.694 |
| ≥ 58 | 31 | 15 | | 27 | 19 | | 37 | 9 | |
| Smoking | | | | | | | | | |
| Never | 6 | 4 | 0.728 | 7 | 3 | 0.737 | 8 | 2 | 1 |
| Ever | 44 | 22 | | 41 | 25 | | 52 | 14 | |
| Alcohol use | | | | | | | | | |
| Low | 19 | 5 | 0.169 | 16 | 4 | 0.206 | 20 | 4 | 0.323 |
| Moderate | 14 | 12 | | 13 | 13 | | 18 | 8 | |
| High | 17 | 9 | | 19 | 7 | | 22 | 4 | |

[a]Statistical analysis performed by Fisher's test or Chi-squared test, where appropriate.
[b]Age, according to median value of 58 years (range 39–80); Met, number of patients with methylated gene promoter; Unmet, number of patients with unmethylated gene promoter; Comethylation, occurrence of hypermethylation of both genes detected in the tumor tissue of patients with tongue carcinoma.

**Table 3** Association of *RUNX3* and *Wnt inhibitory factor 1* (*WIF1*) genes promoter hypermethylation with clinicopathological variables of patients with tongue carcinoma

| *Variables* | *RUNX3 Unmet* | *RUNX3 Met* | P[a] | *WIF1 Unmet* | *WIF1 Met* | P[a] | *Comethylation* – | *Comethylation* + | P[a] |
|---|---|---|---|---|---|---|---|---|---|
| Stage | | | | | | | | | |
| II | 19 | 2 | 0.006 | 16 | 5 | 0.188 | 20 | 1 | 0.055 |
| III | 31 | 24 | | 32 | 23 | | 40 | 15 | |
| Nodal status | | | | | | | | | |
| N 0 | 17 | 2 | 0.013 | 15 | 4 | 0.169 | 18 | 1 | 0.058 |
| N+ | 33 | 24 | | 33 | 24 | | 42 | 15 | |
| Tumor size | | | | | | | | | |
| T1/2 | 40 | 20 | 0.755 | 38 | 22 | 0.951 | 47 | 13 | 1 |
| T3/4 | 10 | 6 | | 10 | 6 | | 13 | 3 | |
| Recurrence | | | | | | | | | |
| No | 24 | 10 | 0.428 | 23 | 11 | 0.465 | 28 | 6 | 0.512 |
| Yes | 26 | 16 | | 25 | 17 | | 32 | 10 | |
| Histologic grade | | | | | | | | | |
| 0 | 17 | 7 | 0.692 | 14 | 10 | 0.722 | 18 | 6 | 0.737 |
| 1 | 25 | 13 | | 24 | 14 | | 30 | 8 | |
| 2 | 8 | 6 | | 10 | 4 | | 12 | 2 | |
| Nuclear grade | | | | | | | | | |
| 0 | 10 | 6 | 0.816 | 11 | 5 | 0.424 | 14 | 2 | 0.156 |
| 1 | 23 | 13 | | 20 | 16 | | 25 | 11 | |
| 2 | 17 | 7 | | 17 | 7 | | 21 | 3 | |

[a]Statistical analysis performed by Fisher's test or Chi-squared test, where appropriate.
Met, number of patients with methylated gene promoter; Unmet, number of patients with unmethylated gene promoter; Comethylation, occurrence of hypermethylation of both genes detected in the tumor tissue of patients with tongue carcinoma.

that methylation should be studied not only on the individual genes but also on the panel of genes that are interacting in the same pathway (Supic *et al*, 2009); thus, we have correlated the occurrence of *RUNX3* and *WIF1* genes comethylation with prognostic parameters, which revealed that in tumors with both genes hypermethylated, a trend of association with nodal status and tumor stage was observed.

In concordance with our findings, previously it has been reported that *RUNX3* was highly methylated in oral cancer (Gao *et al*, 2009) and nasopharyngeal carcinoma (Tan *et al*, 2006), while *WIF1* was found to be silenced by methylation in oral cancer (Pannone *et al*, 2010), nasopharyngeal cancer (Lin *et al*, 2006; Chan *et al*, 2007), and esophageal cancer (Clément *et al*, 2008). Our findings of frequent *WIF1* and *RUNX3*

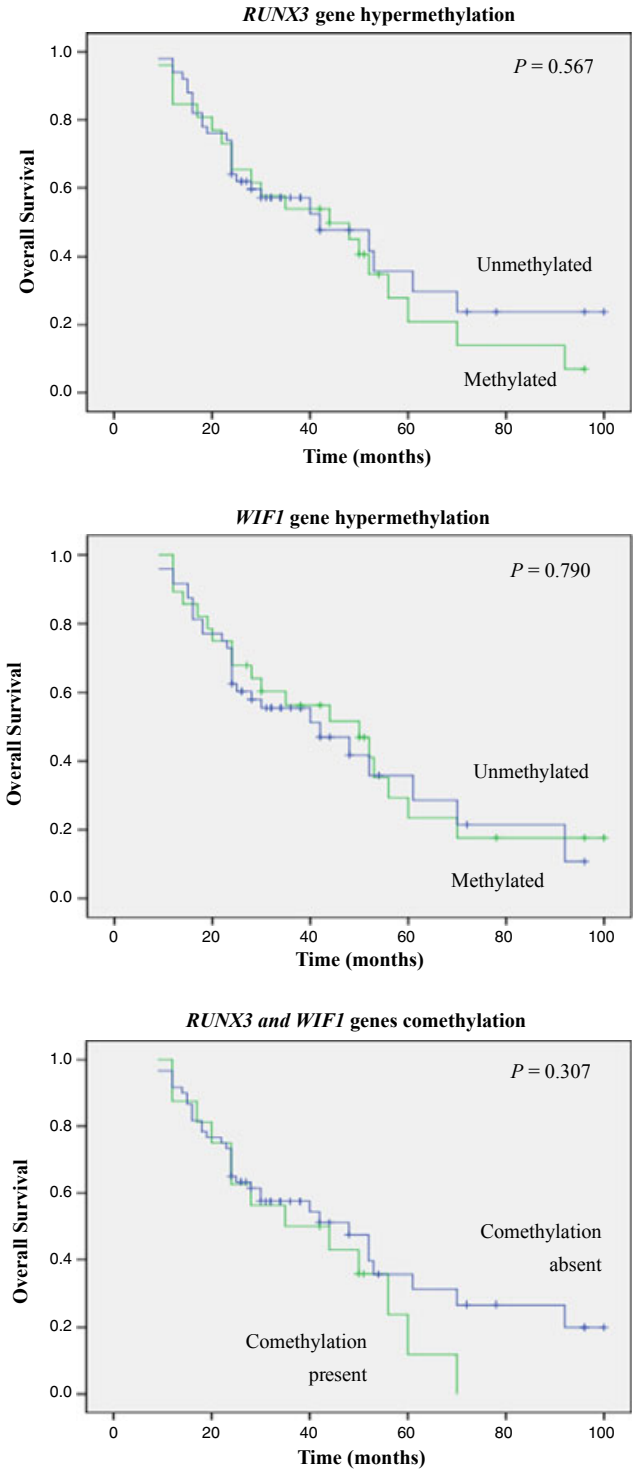


**Figure 2** Kaplan–Meier estimation of overall survival rates among 76 patients with tongue carcinoma. (**a**) Overall survival of patients with *RUNX3* promoter methylated. (**b**) Overall survival of patients with *Wnt inhibitory factor 1* (*WIF1*) gene methylation. (**c**) Overall survival of patients with *RUNX3* and *WIF1* genes comethylation

hypermethylation are also consistent with studies in other cancer types of epithelial origin, which reported promoter methylation as a major mechanism for the inactivation of these tumor suppressor genes. Loss of *RUNX3* expression because of hypermethylation is a common finding in intestinal, colorectal, lung, and breast cancer (Ahlquist *et al*, 2008; Ito *et al*, 2008; Licchesi *et al*, 2008; Park *et al*, 2011). *WIF1* was found to be hypermethylated in lung, breast, bladder, and colon cancer (Wissmann *et al*, 2003; Mazieres *et al*, 2004; Ai *et al*, 2006; Urakami *et al*, 2006; Lee *et al*, 2009).

Positive correlation between methylation of *RUNX3* gene and the presence of lymph nodes metastases and advanced tumor stage, observed in our study, indicates the possible role of this protein in tumor dissemination and its function as a tumor suppressor gene. Hypermethylation of *RUNX3* was independently associated with an increased risk of progression in esophageal carcinoma (Torquati *et al*, 2004; Schulmann *et al*, 2005; Long *et al*, 2007). Methylation level and frequency of *RUNX3* methylation were significantly higher in ductal carcinoma *in situ* and invasive ductal carcinoma than in atypical ductal hyperplasia and flat epithelial atypia of the breast (Park *et al*, 2011). In addition, in patients with non-small-cell lung cancer, *RUNX3* methylation status was found to be an independent prognostic factor for poor survival (Yanagawa *et al*, 2007). In gastric carcinomas, loss of *RUNX3* gene expression caused by promoter methylation could be mediated by an increase in DNA methyltransferase 1 (*DNMT1*), because treatment with demethylating *DNMT1* inhibitor 5′-azadeoxycytidine caused the re-expression of *RUNX3* in gastric cell lines (Chen *et al*, 2010).

The role of *RUNX3* as a possible tumor suppressor in *OSCC* and *HNSCC* is yet controversial. *RUNX3* acts as a tumor suppressor gene in oral carcinoma (Gao *et al*, 2009) and nasopharyngeal carcinoma (Tan *et al*, 2006). However, recent studies with opposite findings revealed that *RUNX3* may have oncogenic role in *HNSCC* when overexpressed, in part because of the altered methylation status (Tsunematsu *et al*, 2009; Kudo *et al*, 2011). It has been revealed that *RUNX3* overexpression promoted cell growth and inhibited apoptosis in head and neck cancer cells (Kudo *et al*, 2011). Moreover, it has been shown that *RUNX3* overexpression in *HNSCC* compared with normal oral mucosa may be caused by demethylation, which increased with head and neck tumor progression (Tsunematsu *et al*, 2009).

It is still unrevealed why *RUNX3* may have antagonistic tumor suppressor/oncogenic role in different types of epithelial cancer and during cancer progression. In contrast to the skin, where basal epidermal cells showed *RUNX3* expression in its nuclei (Salto-Tellez *et al*, 2006), in oral mucosa, only a few normal basal epithelial cells expressed *RUNX3*, while most of head and neck cancer cells expressed *RUNX3* in their nuclei (Tsunematsu *et al*, 2009). This potentially different role of *RUNX3* in *HNSCC* and *OSCC* may be attributed to the distinct pathogenesis of these types of cancer, although both arise from squamous epithelium. Investigation of where *RUNX3* was methylated and underexpressed in normal tissue and demethylated and overexpressed in cancer tissue (Tsunematsu *et al*, 2009) was conducted on *HNSCC*, characterized by

heterogeneous histology and different etiologies, while our study was conduced on homogenous group of patients with tongue carcinoma. Our results of frequent inactivation of *RUNX3* by promoter hypermethylation in tongue carcinoma tissue are in concordance with another study conducted on *OSCC* (Gao *et al*, 2009).

In addition to functional mutations and transcriptional modulation by activators or repressors, a novel mechanism of *RUNX3* tumor suppressor inhibition by the cytoplasmic retention of *RUNX3* protein (protein mislocalization) has been proposed in gastric, breast, and bladder cancer (Ito *et al*, 2005; Kim *et al*, 2005; Lau *et al*, 2006). It has been found that *RUNX3* does not elicit tumor suppressor activity when it is restricted to the cytoplasm (Ito *et al*, 2005). Functional mutations or protein mislocalization was not found in *HNSCC* (Tsunematsu *et al*, 2009), indicating that *RUNX3* is fully functional in *HNSCC* cells. However, frequent occurrence of *RUNX3* protein mislocalization was recently observed in *OSCC* (Gao *et al*, 2009). Thus, differences in methylation status and *RUNX3* tumor suppressor/oncogenic role in *OSCC* and *HNSCC* could be accounted for the occurrence of protein mislocalization.

The finding that the epigenetic silencing of *RUNX3* occurred preferentially in male patients compared with females patients ($P = 0.040$) may be related to etiologic factors because higher percentages of heavy drinkers (37%) and smokers (91%) were observed among male patients as compared to female patients (17% and 70%, respectively). However, methylation status of *RUNX3* or *WIF1* gene was not directly associated with smoking or alcohol use.

A limitation to our study is the lack of evaluation of methylation status in normal oral epithelium and the oral dysplasias of the tongue, because it has been shown that *RUNX3* expression levels were highest in the oral dysplasias, followed by oral squamous carcinoma tissue and normal oral epithelium (Tanji *et al*, 2007). In addition, a source of bias could be methylation analysis in DNA isolated from whole tumor tissue, because oral mucosal tissue usually contains connective tissue and infiltrating lymphocytes. While in normal oral mucosa only a few basal epithelial cells expressed *RUNX3* in their nuclei, in oral mucosa with inflammatory cells infiltration, infiltrating lymphocytes showed high *RUNX3* expression (Tsunematsu *et al*, 2009). Although this is, to our knowledge, the largest study of primary tongue carcinoma in relation to promoter methylation, a possible limitation of this study is relatively small sample size, and the results need to be replicated in larger studies.

In conclusion, our findings indicate that *Wnt* antagonists *RUNX3* and *WIF1* are frequently aberrantly methylated and that *RUNX3* promoter methylation could be considered as a potential molecular marker for the lymph node involvement and the advanced tumor stage in tongue carcinoma. The inactivation of *RUNX3* and *WIF1* by promoter methylation may improve the understanding of the biology of oral cancer and the mechanisms underlying the *Wnt* pathway and may provide new prognostic and therapeutic potentials. Confirmations from larger prospective clinical trials are needed to further establish these observations.

*Ethics statement*

Ethical Committee of Military Medical Academy reviewed this research and approved the investigations on human samples, according to Human Subjects Protection regulations.

## Conflict of interest statement

None declared.

## Author contributions

G. Supic contributed to the conception and design, analysis and interpretation of data, drafting and revising the article, and final approval of the manuscript. R. Kozomara, N. Jovic and K. Zeljic contributed in acquisition, analysis and interpretation of data, and revising the article. Z. Magic contributed to the conception and design, interpretation of data, drafting and revising the article, and final approval of the manuscript.

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