The Clinical Signification of *Claudin-11* Promoter Hypermethylation for Laryngeal Squamous Cell Carcinoma

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ABCDEFG 1 **Zhisen Shen**
BCDEF 2 **Bing Cao**
ABCDE 2 **Lexi Lin**
BCD 2 **Chongchang Zhou**
BCD 1 **Dong Ye**
BCD 1 **Shijie Qiu**
BD 1 **Qun Li**
BD 1 **Xiang Cui**

1 Department of Otolaryngology (Head and Neck Surgery), Ningbo Medical Center Lihuili Hospital Affiliated to Ningbo University, Ningbo, Zhejiang, P.R. China;
2 Department of Otolaryngology, School of Medicine, Ningbo University, Ningbo, Zhejiang, P.R. China

Corresponding Author:
Zhisen Shen, e-mail: szs7216@163.com

Background: *Claudin-11 (CLDN11)* is frequently silenced by its promoter hypermethylation. Previous studies have shown that *CLDN11* promoter hypermethylation is a potential biomarker for diagnosing various cancers. The aim of this study was to investigate *CLDN11* promoter methylation and its potential relevance to clinicopathologic features and prognosis of patients with laryngeal squamous cell carcinoma (LSCC).

Material/Methods: Using the quantitative methylation-specific polymerase chain reaction (qMSP), *CLDN11* promoter methylation was measured in 91 tumor tissues and their paired adjacent normal tissues, and the relationship between *CLDN11* methylation and clinicopathologic features was evaluated. A receiver operating characteristic (ROC) curve was created to assess diagnostic values, and the Kaplan-Meier survival analysis was used to evaluate the association between *CLDN11* methylation and prognosis of patients with LSCC.

Results: Our results showed significantly elevated promoter methylation of *CLDN11* in tumor tissues compared to their adjacent tissues (p=1.227E-16). *CLDN11* promoter methylation also increased in patients with lymph node metastasis (p=0.009), advanced clinical stage (p=9.26E-06) and higher T classification (p=0.003). The area under the ROC curve (AUC) of *CLDN11* was 0.884 (95% CI=0.835–0.932, p<0.01). The Kaplan-Meier analysis indicated that high *CLDN11* promoter methylation levels were associated with poor overall survival of LSCC patients (log-rank test, p=0.007).

Conclusions: We demonstrated that *CLDN11* promoter hypermethylation is a frequent event in LSCC, and contributes to metastasis and progression of LSCC. Thus, *CLDN11* could be a potential biomarker for diagnosis and prognosis of LSCC patients.

MeSH Keywords: Biological Markers • Claudins • DNA Methylation • Laryngeal Neoplasms

Full-text PDF: http://www.medscimonit.com/abstract/index/idArt/904751

📄 2159   🗐 1   📊 3   📖 47

Background

Laryngeal cancer is the second most common cancer among respiratory system tumors. According to a report by the American Cancer Society, 13,360 new laryngeal cancers and 3,660 new deaths were projected to occur in the USA in 2017 [1]. Laryngeal squamous cell carcinoma (LSCC) is the most common pathological type of laryngeal cancer [2]. In China, there has been an increasing incidence of laryngeal cancer in the younger population, especially in the northern region of China [3]. Despite remarkable advances in diagnostic methods and therapeutic strategies for LSCC, the 5-year survival rate has decreased over the past several decades in the USA, and the quality of life after surgical treatment is greatly impacted by pain, dry mouth, and speech disorders [1,4].

The pathogenesis of LSCC is a multifactorial process involving a variety of complicated factors, including epigenetic and genetic alterations, as well as environmental exposure to tobacco, alcohol, asbestos, and viral infection [5,6]. Studying epigenetics, especially aberrant promoter methylation of tumor suppressor genes (TSGs), which is the most common and best-characterized alteration, offers great potential for identifying biomarkers that can be used in the diagnosis and prognosis of LSCC patients [7]. There is a growing list of TSGs that are silenced or inactivated by promoter hypermethylation, which is considered to be a significant event in the initiation and progression of various cancers [8–11]. *Claudin-11 (CLDN11)* is a potential tumor suppressor gene located at chromosome 3 (3q26.2), and encodes a member of the claudin family, an integral membrane protein of the paracellular tight junction (TJ) [12,13]. TJs are often altered in human carcinomas, and the loss of TJs has been interpreted as an important step in cancer progression [14]. Moreover, TJs play a number of diverse roles in tumorigenesis [15±–18]. Previous studies have demonstrated that silencing of *CLDN11* expression is associated with increased invasiveness in various cancers [19–21]. In addition, Soini et al. [22] reported that the poor outcome of patients with meningiomas was associated with reduced *CLDN11* expression. *CLDN11* acts as a candidate tumor suppressor gene whose expression is frequently diminished or silenced by its promoter hypermethylation in gastric cancer and malignant melanoma [19,23]. Moreover, a previous study reported that aberrant promoter methylation of *CLDN11* could be associated with the malignant transformation of oral leukoplakia [24]. In addition, hypermethylation of *CLDN11* frequently occurs in malignant melanoma, and can be used in a clinical setting to distinguish malignant melanocytic lesions from benign ones [23].

However, there are few studies that focus on the role of *CLDN11* promoter methylation in LSCC. To the best of our knowledge, this is the first study to investigate the methylation status of *CLDN11* in LSCC, and to correlate this methylation with clinical parameters, as well as overall survival of LSCC patients. We hypothesized that *CLDN11* promoter hypermethylation is a potential biomarker for diagnosis and prognosis of this disease.

Material and Methods

Patients and collection of tissue samples

Tumor tissues and their paired adjacent normal tissues (dissected at >0.5 cm from the margin of the neoplastic lesion) were collected from 91 LSCC patients comprising 87 males and four females, all who had undergone surgical operation at the Department of Otolaryngology of Ningbo Medical Center Lihuili Hospital, Zhejiang, China. The age of the patients ranged from 40 to 86 years, and the median age was 60 years. The majority of patients (80%) were cigarette smokers. No chemotherapy or radiotherapy had been administered to these patients prior to surgical intervention, and none of the patients had a prior history of cancer. Histological confirmation of tumors was performed by two pathologists according to the Union for International Cancer Control (UICC) classification (TNM 2002). There were 78 well or moderately differentiated cases and 13 poorly differentiated cases. Furthermore, there were 44 stage I/II and 47 stage III/IV cases. According to the postoperative pathology, there were 32 cases with lymph node metastasis and 59 cases without lymph node metastasis. All samples were frozen immediately in liquid nitrogen and stored at -80°C until required. All participants signed an informed consent form. The study received approval from the local Ethical Committee at the Department of Otolaryngology at Ningbo Medical Center Lihuili Hospital, Zhejiang, China.

DNA extraction and bisulfite modification

Genomic DNA was extracted from tissue samples using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA quality and quantity were assessed using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Co. Ltd., Wilmington, USA). Eluted DNA was subjected to bisulfite modification using a Methylamp DNA modification kit with ZYMO EZ DNA Methylation-Gold Kit according to the manufacturer's instructions (Zymo Research, Orange, CA, USA).

Quantitative methylation-specific polymerase chain reaction (qMSP)

qMSP was used to determine the DNA methylation status of the *CLDN11* promoter. The bisulfite-treated DNA was amplified using a LightCycler 480 (Roche Diagnostics, Mannheim, Germany) according to the instrument guidelines. The

primer sequences used in this experiment were *CLDN11*-Forward 5'-ATAAGTTGATAGGAGAATCGAATCG-3', *CLDN11*-Reverse 5'-AACGAAAATAACCCTAAACACGTA-3'. *ACTB*-Forward 5'-TGGTGATGGAGGAGGTTTAGTAAGT-3', *ACTB*-Reverse 5'-AACCAATAAAACCTACTCCTCCCTTAA-3'. The polymerase chain reaction (PyroMark PCR Kit; Qiagen) amplification consisted of 50 cycles (of 95°C for 20 seconds, 60°C for 30 seconds and 72°C for 30 seconds) after an initial denaturation step (at 95°C for 10 minutes). The Bisulfite-Converted Universal Methylated Human DNA Standard (ZYMO Research Company) was used as a positive control. To correct for differences in both quality and quantity between samples, *ACTB* was used as an internal control. Levels of methylation were calculated by percentage of methylated reference (PMR), PMR=$2^{-\Delta\Delta Ct} \times 100\%$, in which $\Delta\Delta Ct = (Ct_{sample} - Ct_{internal\ control}) - (Ct_{positive\ control} - Ct_{internal\ control})$.

Statistical analyses

All statistical analyses were performed using the SPSS software v18.0 (SPSS Inc., Chicago, IL, USA). Paired sample *t*-tests were used to compare *CLDN11* promoter methylation levels between tumor tissues and their paired adjacent normal tissues. Analysis of variance (ANOVA) was used to evaluate the association between *CLDN11* methylation and clinicopathologic features, including gender, age, smoking behavior, histological classification, T classification, lymph node metastasis, and clinical stage. Overall survival curves were calculated by Kaplan-Meier analysis and the differences between curves were evaluated using the log-rank test. A *p* value <0.05 was considered to be statistically significant. All figures were created using the GraphPad Prism 6 software (GraphPad, San Diego, CA, USA).

Results

In the present study, we measured the *CLDN11* promoter methylation levels in 91 LSCC tissues and their adjacent non-tumor tissues using qMSP. Results indicated that methylation levels of *CLDN11* in tumor tissues were significantly higher than in the paired tissues (*p*=1.227E-16, Figure 1).

Previous studies have shown that abnormal promoter methylation correlates with clinicopathological characteristics in numerous cancers, such as colorectal cancer [25], oral cancer [26], bladder cancer [27], and gastric cancer [28]. We therefore investigated the correlation of *CLDN11* methylation with the clinicopathological features of LSCC patients, such as gender, age, smoking behavior, histological classification, T classification, lymph node metastasis, and clinical stage. As shown in Table 1, methylation levels of *CLDN11* in patients with lymph node metastasis were significantly higher than in patients without lymph node metastasis (*p*=0.009). When stratified for TNM
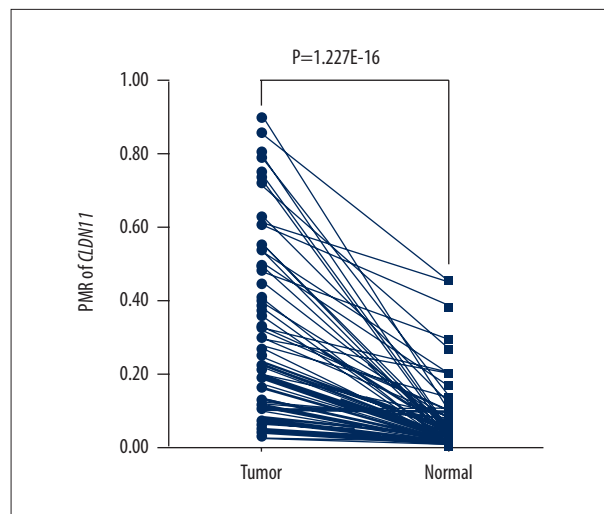

Figure 1. Comparison of methylation levels of *CLDN11* between tumor tissues and paired adjacent normal tissues. There was a significant difference in methylation of the *CLDN11* promoter in LSCC tissues compared to normal tissues (N=91).

stages, *CLDN11* methylation in patients with advanced stages of LSCC was remarkably elevated compared to early stages (*p*=9.26E-06). In addition, we found a marked increase in *CLDN11* methylation in patients with T3+4 compared to T1+2 (*p*=0.003). However, there was no statistically significant association between *CLDN11* methylation and other clinicopathological features, such as age, smoking behavior, or histological classification.

To further evaluate the diagnostic significance of *CLDN11*, we then constructed a ROC curve by plotting sensitivity versus specificity (Figure 2). Results showed that the AUC of *CLDN11* was up to 0.884 (95% CI=0.835–0.932, *p*<0.01). Based on the maximum Youden index, distinguishing between patients with LSCC from controls, the cutoff point of methylation frequency was 0.571, at which the sensitivity and specificity for predicting LSCC were 92.30% and 73.60%, respectively. Our results demonstrated that the methylation level of the majority of tumor tissues was less than the cutoff value. In addition, we performed Kaplan-Meier analysis to evaluate the association of methylated *CLDN11* with overall survival. We used the mean methylation level of tumor tissues to classify patients into two groups: 30 patients were placed into the hypermethylation group, and the remaining patients were categorized as the hypomethylation group [29]. Kaplan-Meier analysis indicated that patients with higher methylation frequency tended to have a poorer overall survival compared to patients with lower methylation (log-rank test, *p*=0.007, Figure 3).
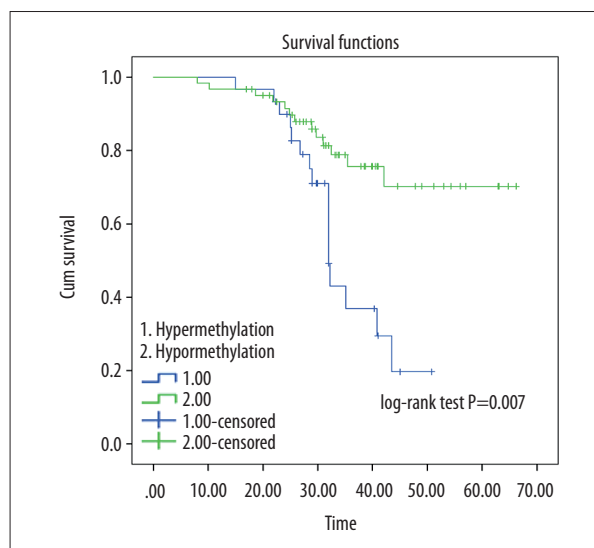
Table 1. Clinicopathologic features of patients with LSCC, and correlation between the methylation status of *CLDN11* and these features.

Features	Variables	No.	Mean ±SD	*P* value
Gender	Female	4	0.164±0.101	0.393
	Male	87	0.260±0.223	
Age	<60 y	46	0.254±0.203	0.917
	≥60 y	45	0.259±0.237	
Smoking behavior	No	18	0.259±0.203	0.955
	Yes	73	0.256±0.224	
Histological classification	Well/moderately	78	0.258±0.219	0.824
	Poorly	13	0.244±0.229	
Clinical stage	Stage I+II	44	0.155±0.099	9.26E-06
	Stage III+IV	47	0.351±0.257	
T classification	T1+2	55	0.196±0.168	0.003
	T3+4	36	0.348±0.256	
Lymph metastasis	No	59	0.213±0.193	0.009
	Yes	32	0.337±0.245	
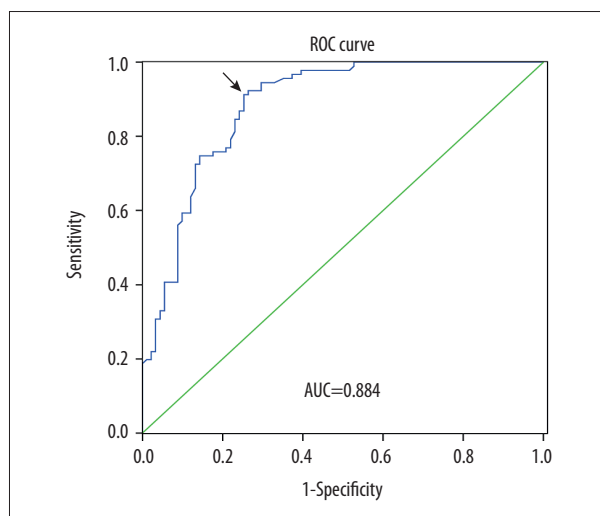
Figure 2. Receiver operating characteristic (ROC) analyses of the curve (N=91). The cutoff point was defined as the maximum Youden index, which is highlighted by the arrow.

Figure 3. Kaplan-Meier curves of overall survival of LSCC patients in the *CLDN11* hypermethylation group and the *CLDN11* hypomethylation group. The log-rank test result showed that patients with *CLDN11* hypermethylation had a poor overall survival (*p*=0.007).

Discussion

Loss of claudins may reduce cell-cell adhesion, which offers a selective advantage to developing tumor cells [30]. Previous studies have shown that *claudin-11* is downregulated in a variety of cancers [19–21]. In mouse skin tumorigenesis, expression of *CLDN11* also decreased during tumor progression [31]. In the current study, we investigated the association of *CLDN11* with LSCC.

There are few typical clinical symptoms of the early stage of laryngeal cancer, especially in supraglottic cancer [32]. Patients with laryngeal cancer are usually diagnosed by computerized tomography (CT), magnetic resonance imaging (MRI) and endoscopic biopsy [33]. However, the sensitivity and specificity of imaging techniques are unsatisfactory [34,35], and very few patients with laryngeal cancer are diagnosed through

conventional imaging techniques [3]. Biopsy remains the gold standard for diagnosing laryngeal cancer [36]. This approach, however, is limited due to its invasiveness, and is not suitable for large-scale screening. Therefore, there is an urgent need to further investigate molecular mechanisms and to develop potential biomarkers to improve early diagnosis of this disease. Accumulating studies have reported other biomarkers in LSCC, including CD133 [37], P53, Bcl-2, and caspase-3 [38]. Here, we provided another biomarker (CLDN11) in LSCC.

Abnormal promoter methylation of TSGs may be an early event in tumorigenesis, and thus a warning signal for the onset of LSCC [39]. In recent years, *CLDN11* methylation has been reported to be important in tumor occurrence and development. Agarwal et al. [19] found that *CLDN11* methylation was elevated in gastric cancer tissues compared to their matching gastric mucosal tissues, and promoter hypermethylation of *CLDN11* has been shown to decrease gene expression, resulting in increased cell motility and invasion. In addition, Walesch et al. [23] reported a significant increase in the methylation level of *CLDN11* in malignant melanoma metastases compared to primary malignant melanoma and nevus cell nevi. Furthermore, Abe et al. [24] hold the view that aberrant promoter methylation of *CLDN11* is associated with high-risk oral leukoplakia. In our study, a significant increase in *CLDN11* promoter methylation was found in LSCC tissues compared to non-cancerous tissues, suggesting that *CLDN11* methylation is a frequent tumor-specific event in LSCC, and could serve as a potential biomarker for the detection of LSCC.

Lymph metastasis, clinical stage, and T classification are important prognostic indicators in patients with squamous cell carcinoma of the head and neck, which influence survival rates and determine the treatment modality [39–42]. Moreover, patients with advanced stage laryngeal cancer who receive total laryngectomy followed by chemoradiation therapy experience many kinds of discomfort [43,44]. Our study also found that *CLDN11* methylation was remarkably higher in patients with lymph node metastasis, advanced clinical stage, and higher T classification, in agreement with previous studies, suggesting that *CLDN11* could play a crucial role in disease progression and metastasis.

Regarding head and neck cancers, several tumor markers have been evaluated in an attempt to improve diagnosis and follow-up, including carcinoembryonic antigen (CEA), tissue polypeptide antigen (TPA-M), squamous cell carcinoma antigen (SCCA) and cytokeratin 19 fragments (CYFRA 21-1). Eleftheriadou et al. [45] reported that the sensitivity of CEA, TPA-M, SCCA, and CYFRA 21-1 in the diagnosis of laryngeal cancer was from 16.5% to 46.8%. In our study, *CLDN11* had a higher sensitivity and specificity in the screening of laryngeal cancer, with an AUC of 0.884, sensitivity of 92.30%, and specificity of 73.60%, suggesting that *CLDN11* methylation could have a higher diagnostic value in distinguishing patients with LSCC from controls.

Previous quantitative reviews have shown that the expression of *CLDN11* significantly correlated with survival of breast cancer patients [46]. Multiple factors regulate gene transcription, and aberrant DNA methylation is one of the most important mechanisms [47]. In our study, methylation frequencies were found to be negatively related to the prognosis of LSCC, suggesting that aberrant methylated *CLDN11* could be a novel biomarker for predicting the prognosis of patients with LSCC, resulting in a massive impact on clinical decision making.

However, there are some limitations of this research. First, our cohort consisted of only 91 patients with LSCC; a greater number of samples are needed to identify patients with a higher predictive capability. Second, due to the limited amount of tissues, we were unable to have matched gene expression. The expression of *CLDN11* should be further investigated, along with the association between *CLDN11* promoter methylation and expression.

Conclusions

Our study provided evidence that *CLDN11* promoter methylation occurred frequently in LSCC, and was significantly correlated with lymph node metastasis, advanced clinical stage, and higher T classification. In addition, patients with *CLDN11* hypermethylation have poor overall survival. These results suggest that *CLDN11* hypermethylation could be a potential biomarker for diagnosis and prognosis of LSCC.

Conflicts of interest

None.

References:

1. Siegel RL, Miller KD, Jemal A: Cancer statistics, 2017. Cancer J Clin, 2017; 67(1): 7–30
2. Morshed K, Polz-Dacewicz M, Szymański M et al: Short-fragment PCR assay for highly sensitive broad-spectrum detection of human papillomaviruses in laryngeal squamous cell carcinoma and normal mucosa: Clinicopathological evaluation. Eur Arch Otorhinolaryngol, 2008; 265: S89–96
3. Chen K, Song F, He M et al: Trends in head and neck cancer incidence in Tianjin, China, between 1981 and 2002. Head Neck, 2009; 31(2): 175–82
4. Luo J, Wu J, Lv K et al: Analysis of postsurgical health-related quality of life and quality of voice of patients with laryngeal carcinoma. Medicine (Baltimore), 2016; 95(1): e2363

5. Muscat JE, Wynder EL: Tobacco, alcohol, asbestos, and occupational risk factors for laryngeal cancer. Cancer, 1992; 69(9): 2244–51

6. Yu PJ, Chen WG, Feng QL et al: Association between *CYP1B1* gene polymorphisms and risk factors and susceptibility to laryngeal cancer. Med Sci Monit, 2015; 21: 239–45

7. Choudhury JH, Ghosh SK: Promoter hypermethylation profiling identifies subtypes of head and neck cancer with distinct viral, environmental, genetic and survival characteristics. PLoS One, 2015; 10(6): e0129808

8. Li QL, Ito K, Sakakura C et al: Causal relationship between the loss of RUNX3 expression and gastric cancer. Cell, 2002; 109(1): 113–24

9. Herman D, Leakey TI, Behrens A et al: *CHST11* gene expression and DNA methylation in breast cancer. Int J Oncol, 2015; 46(3): 1243–51

10. Grageda M, Silveyra P, Thomas NJ et al. DNA methylation profile and expression of surfactant protein A2 gene in lung cancer. Exp Lung Res, 2015; 41(2): 93–102

11. Iwata N1, Ishikawa T, Okazaki S et al: Clinical significance of methylation and reduced expression of the quaking gene in colorectal cancer. Anticancer Res, 2017; 37(2): 489–98

12. Furuse M, Fujita K, Hiiragi T et al: Claudin-1 and -2: novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin. J Cell Biol, 1998; 141(7): 1539–50

13. Morita K, Sasaki H, Fujimoto K et al: Claudin-11/OSP-based tight junctions of myelin sheaths in brain and Sertoli cells in testis. J Cell Biol, 1999; 145(3): 579–88

14. Morin PJ: Claudin proteins in human cancer: Promising new targets for diagnosis and therapy. Cancer Res, 2005; 65(21): 9603–6

15. Tsukita S, Furuse M, Itoh M: Multifunctional strands in tight junctions. Nat Rev Mol Cell Biol, 2001; 2(4): 285–93

16. Köhler K, Zahraoui A: Tight junction: A co-ordinator of cell signalling and membrane trafficking. Biol Cell, 2005; 97(8): 659–65

17. Matter K, Balda MS: Signalling to and from tight junctions. Nat Rev Mol Cell Biol, 2003; 4(3): 225–36

18. Balda MS, Garrett MD, Matter K: The ZO-1-associated Y-box factor ZONAB regulates epithelial cell proliferation and cell density. J Cell Biol, 2003; 160(3): 423–32

19. Agarwal R, Mori Y, Cheng Y et al: Silencing of claudin-11 is associated with increased invasiveness of gastric cancer cells. PLoS One, 2009; 4(11): e8002

20. Awsare NS, Martin TA, Haynes MD et al: Claudin-11 decreases the invasiveness of bladder cancer cells. Oncol Rep, 2011; 25(6): 1503–9

21. Nissinen L, Siljamäki E, Riihilä P et al: Expression of claudin-11 by tumor cells in cutaneous squamous cell carcinoma is dependent on the activity of p38δ. Exp Dermatol, 2016 [Epub ahead of print]

22. Soini Y, Rauramaa T, Alafuzoff I et al: Claudins 1, 11 and twist in meningiomas. Histopathology, 2010; 56(6): 821–24

23. Walesch SK, Richter AM, Helmbold P et al: Claudin11 promoter hypermethylation is frequent in malignant melanoma of the skin, but uncommon in nevus cell nevi. Cancers (Basel), 2015; 7(3): 1233–43

24. Abe M, Yamashita S, Mori Y et al: High-risk oral leukoplakia is associated with aberrant promoter methylation of multiple genes. BMC Cancer, 2016; 16: 350

25. Vedeld HM, Skotheim RI, Lothe RA: The recently suggested intestinal cancer stem cell marker DCLK1 is an epigenetic biomarker for colorectal cancer. Epigenetics, 2014; 9(3): 346–50

26. Schussel JL, Kalinke LP, Sassi LM et al: Expression and epigenetic regulation of DACT1 and DACT2 in oral squamous cell carcinoma. Cancer Biomark, 2015; 15(1): 11–17

27. Chung W, Bondaruk J, Jelinek J et al: Detection of bladder cancer using novel DNA methylation biomarkers in urine sediments. Cancer Epidemiol Biomarkers Prev, 2011; 20(7): 1483–91

28. Hibi K, Goto T, Shirahata A et al: Detection of TFPI2 methylation in the serum of gastric cancer patients. Anticancer Res, 2011; 31(11): 3835–38

29. Wang ZR, Wei JH, Zhou JC et al: Validation of DAB2IP methylation and its relative significance in predicting outcome in renal cell carcinoma. Oncotarget, 2016; 7(21): 31508–19

30. Mullin JM: Potential interplay between luminal growth factors and increased tight junction permeability in epithelial carcinogenesis. J Exp Zool, 1997; 279(5): 484–89

31. Arabzadeh A, Troy TC, Turksen K: Changes in the distribution pattern of Claudin tight junction proteins during the progression of mouse skin tumorigenesis. BMC Cancer, 2007; 7: 196

32. Brouha XD, Tromp DM, de Leeuw JR et al: Laryngeal cancer patients: Analysis of patient delay at different tumor stages. Head Neck, 2005; 27(4): 289–95

33. Joshi VM, Wadhwa V, Mukherji SK: Imaging in laryngeal cancers. Indian J Radiol Imaging, 2012; 22(3): 209–26

34. Allegra E, Ferrise P, Trapasso S et al: Early glottic cancer: Role of MRI in the preoperative staging. Biomed Res Int, 2014; 2014: 890385

35. Becker M, Zbären P, Laeng H et al: Neoplastic invasion of the laryngeal cartilage: Comparison of MR imaging and CT with histopathologic correlation. Radiology, 1995; 194(3): 661–69

36. Jovanović MB: Diagnosis of laryngeal carcinoma. Med Pregl, 2008; 61(11–12): 591–95

37. Qiu HO, Wang H, Che N et al: Identification and characterization of CD133(pos) subpopulation cells from a human laryngeal cancer cell line. Med Sci Monit, 2016; 22: 1146–51

38. Yao C, Cao X, Fu Z et al: Boschniakia rossica polysaccharide triggers laryngeal carcinoma cell apoptosis by regulating expression of Bcl-2, Caspase-3, and P53. Med Sci Monit, 2017; 23: 2059–64

39. Starska K, Forma E, Lewy-Trenda I: Diagnostic impact of promoter methylation and E-cadherin gene and protein expression levels in laryngeal carcinoma. Contemp Oncol (Pozn), 2013; 17(3): 263–71

40. Baatenburg de Jong RJ, Hermans J, Molenaar J: Prediction of survival in patients with head and neck cancer. Head Neck, 2001; 23(9): 718–24

41. Rhee D, Wenig BM, Smith RV: The significance of immunohistochemically demonstrated nodal micrometastases in patients with squamous cell carcinoma of the head and neck. Laryngoscope, 2002; 112(11): 1970–74

42. Nutting CM, Robinson M, Birchall M: Survival from laryngeal cancer in England and Wales up to 2001. Br J Cancer, 2008; 99(Suppl. 1): S38–39

43. Cohen SM, Garrett CG, Dupont WD et al: Voice-related quality of life in T1 glottic cancer: Irradiation versus endoscopic excision. Ann Otol Rhinol Laryngol, 2006; 115(8): 581–86

44. Hanna E, Sherman A, Cash D: Quality of life for patients following total laryngectomy *vs.* chemoradiation for laryngeal preservation. Arch Otolaryngol Head Neck Surg, 2004; 130(7): 875–79

45. Eleftheriadou A, Chalastras T, Ferekidou E: Clinical effectiveness of tumor markers in squamous cell carcinoma of the larynx. Anticancer Res, 2006; 26(3B): 2493–97

46. Meng L, Xu Y, Xu C: Biomarker discovery to improve prediction of breast cancer survival: Using gene expression profiling, meta-analysis, and tissue validation. Onco Targets Ther, 2016; 9: 6177–85

47. Das PM, Singal R: DNA methylation and cancer. J Clin Oncol, 2004; 22(22): 4632–42