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Promoter hypermethylation of tumour suppressor genes in tumour cells from patients with head and neck cancer

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Head and neck cancer is a group of malignancies of the upper respiratory tract, its main risk factors being attributed to tobacco and alcohol consumption and human papillomavirus (HPV) infection. Promoter hypermethylation is one of the major mechanisms in the transcriptional inactivation of tumour suppressor genes (TSGs) in human cancer. In this study, aberrant methylation in the promoter regions of five TSGs and the prevalence of HPV was analysed in order to evaluate the role of epigenetic changes in head and neck carcinogenesis, and to clarify its causative relation with HPV infection and smoking behaviour. Brushing specimens collected from the tumour or oral mucosa from 38 cases with head and neck cancer and 42 controls were included in the study and tested for HPV infection. 34% of cancer patients and 12% of controls were infected by HPV. Brushings from 31 tumours and 11 control samples were analysed by means of methylation-specific PCR. Hypermethylation of at least one gene was detected in 71% of tumours. Epigenetic changes were more frequent in smokers than in never-smokers ($p = 0.047$), and in cancer cases hypermethylation was more prevalent in late-stage tumours. The study shows a significant role of epigenetic changes in head and neck carcinogenesis. Analysis of promoter hypermethylation of selected TSGs in oral brushing specimens from high-risk cases can serve as a simple method for early prediction of head and neck cancer risk.

Key words: head and neck cancer, tumour suppressor genes, DNA hypermethylation, HPV

INTRODUCTION

Head and neck cancer is a group of malignancies affecting functionally and anatomically related organs with a strong association to tobacco use and alcohol consumption. The incidence rates of head and neck squamous cell carcinoma (HNSCC) are high in wide regions of Central and Eastern Europe, including Lithuania [1, 2]. Recent increases of oral and pharyngeal cancers have been reported in Eastern Europe and in Japan [3]. Epidemiological data show that various risk factors are implicated in the carcinogenesis of HNSCC. The main risk factors of HNSCC are attributed to alcohol usage and tobacco smoking [4]. Only a small proportion (approximately 15–20%) of HNSCC develops in non-smokers and non-drinkers. Genetic variation in individual response to tobacco carcinogens is an important factor modifying susceptibility to HNSCC. For instance, polymorphism in the carcinogen metabolizing enzyme CYP2A6 has a significant impact on nasopharyngeal cancer susceptibility [5]. Recent molecular and epidemiological data suggest that human papillomavirus (HPV) infection of the upper airway may play an important role in head and neck carcinogenesis. High risk HPV (HPV 16 and 18) are detected in the HNSCCs, while low-risk HPV (HPV 6 and 11) in respiratory papillomas [6]. Hypermethylation of CpG islands within the promoter region of tumour suppressor genes (TSGs) is an early and frequent event in human carcinogenesis, causing transcriptional inactivation of various genes involved in the regulation of cell growth, differentiation, death and other activities [7]. Recent studies revealed a significance of TSGs hypermethylation in the pathogenesis of HNSCC [8–12], as well as in other tumours.

The study aimed to provide a further insight into the mechanisms by which smoking and HPV infection participate in the development of HNSCC. Novel molecular biomarkers and low-invasive means of analysis are highly important for risk assessment and HNSCC prevention. In order to better understand the role of epigenetic alterations in tobacco exposure and HPV infection-related head and neck carcinogenesis, we examined the frequency of epigenetic changes in five TSGs, namely *p16*, *p14*, *RAR*β, *RASSF1A*, and *MGMT*, in brushing specimens obtained mainly from smokers with and without HNSCC. The genes selected for the study are involved in different pathways, including cell cycle, signaling, DNA repair and apoptosis, and are frequently deregulated during carcinogenesis. The study is an extension of our previous studies on the frequency and significance of HPV infection and epigenetic changes in pathogenesis of different tumours [13, 14].

PATIENTS AND METHODS

Study population and sample collection

Thirty-eight patients from Institute of Oncology, Vilnius University, with primary head and neck cancer were included in

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the analysis (period from March to November 2006). The diagnosis of cancer was done according to histology. As a control group, 42 healthy volunteers were invited to participate in the study. The study protocol was approved by the Lithuanian Bioethics Committee (23.02.2006, No 7), all participants gave signed informed consent to participate in the study. Clinical information, including tumour location, stage, nodal status and metastases (TNM classification), was obtained from the case-history. Smoking status and alcohol consumption were analysed using a questionnaire.

For cancer cases, scrapes of fresh tumour cells were obtained at the moment of surgical intervention. Scrapes of oral mucosa were collected from control cases. For cell collection, sterile brush swabs were used; the end of the brush with cells was collected in 0.5 ml transport PBS solution. HPV testing and typing was performed from fresh (not frozen) material at the Institute of Oncology, Vilnius University. For epigenetic analysis, DNA of good quality was available from 31 HNSCC patients and 11 healthy controls. Only these cases with a complete set of data on HPV infection and TSGs hypermethylation are analysed in detail in the present study. The main characteristics of this study group are summarized in Table 1.

Table 1. **Main characteristics of study groups**[a]

Characteristics	Head and neck cancer patients (n = 31)	Healthy controls (n = 11)
Age		
<60	14 (45)	8 (72)
≥60	17 (55)	3 (27)
Sex		
Female	3 (10)	7 (64)
Male	28 (90)	4 (36)
Smoking status[b]		
Current smoker	27 (87)	3 (27)
Never smoker	4 (13)	5 (45)
HPV status		
Positive	12 (39)	1 (9)
Negative	19 (61)	10 (91)
Tumour stage		
T1-T2	12 (39)	–
T3-T4	19 (61)	–
Tumour localization		
Cutis of head and neck region	5 (16)	–
Larynx and hypopharynx	14 (45)	–
Other localizations[c]	12 (39)	–

[a] Characteristics are presented as mean (%); [b] data on smoking status are lacking for 3 control cases; [c] other localizations include oropharynx, lingua and sinus maxillae.

HPV analysis using DNA amplification

Polymerase chain reaction with consensus primers MY09/MY11 was used to detect HPV. DNA was extracted using the column method (SorpoClean™ Genomic DNA Extracion Module, SORPO Diagnostics, Lithuania) according to the manufacturer's protocol. PCR was performed using REDTaq® ReadyMix™ PCR Reaction Mix with $MgCl_2$ (Sigma). The composition of the PCR mix: 20 mM Tris-HCl (pH 8.3), with 100 mM KCl, 3 mM $MgCl_2$, 0.002% gelatin, 0.4 mM dNTP mix, 50 pmol of each primer and 0.06 unit/μl of Taq DNA polymerase. PCR was performed starting from the initial denaturation step at 93 °C for 3 min, followed by 40 cycles of the denaturation step at 94 °C for 1 min, the primer annealing step at 55 °C for 1 min and a chain elongation step at 72 °C for 1 min and 30 s. A final extension for 5 min at 72 °C was used. For HPV typing, the multiplex PCR with the Ready to use Master Mixes for HPV 6, 11 and for HPV 16, 18 (SORPO Diagnostics, Lithuania) was used.

Amplified PCR products were analysed by electrophoresis in 2% agarosis gel stained with ethidium bromide and visualized in UV light transiluminator (Herolab, Germany).

Methylation specific PCR

Methylation specific PCR (MSP) [15] was used for analysis of the methylation pattern in the 5' region of selected TSGs as described earlier [14]. Briefly, 1 μg of DNA in a volume of 50 μl was denatured with 3 M sodium hydroxide (Sigma) for 15 min at 37 °C and modified with freshly prepared 3.2 M sodium metabisulfite (Sigma) and 10 mM hydroquinone (Sigma) at 50 °C for 16 h. Modified DNA was purified using the Wizard DNA Clean-up System in vacuum manifold according to manufacturer (Promega), followed by 3 M sodium hydroxide treatment, precipitation with 96% ethanol and dissolution in 40 μl of sterile water.

Modified genomic DNA served as a template for MSP. PCR primer pairs specific for methylated (M) and unmethylated (U) sequences within the 5' region of selected genes were synthesized according to published sequences [15–19]. The PCR mixture contained RNase-free water, 2.5 mM $MgCl_2$, 0.4 mM dNTP mixture, 1.25 U AmpliTaq Gold polymerase (Perkin–Elmer), 1 × GeneAmp PGR Buffer, 10 μl/ml dimethyl sulfoxide, 1 μM of each primer and bisulfite converted DNA, in the final volume of 50 μl. Amplification was carried out in an Eppendorf thermocycler in the following conditions: hot start at 95 °C for 10 min, denaturation at 95 °C for 45 s, annealing 63 °C, 64 °C, 63 °C, 59 °C and 65 °C for *p16*, *p14*, *RASSF1A*, *RAR*β and *MGMT* genes, respectively, primer extension at 72 °C for 45 s, and final extension at 72°C for 8 min. The number of cycles was specific of each gene and did not exceed 38.

Each PCR reaction was performed together with controls specific for methylated (KM, *in vitro* methylated leukocyte DNA using bacterial SssI methylase; New England BioLabs) and non-methylated (KL, leukocyte DNA from healthy controls) reaction and RNase-free water. All positive reactions were repeated at least twice. 12 μl of each MSP product was directly loaded onto non-denaturing 7.5% polyacrylamide gel, stained with ethidium bromide and visualized under UV illumination.

Statistical analysis

Data on particular gene's hypermethylation were summarized as the frequencies and percentage. The two-sided Fisher's exact test was used for a comparison of categorical variables. $P \leq 0.05$ was considered statistically significant. Odds ratios (OR) and the exact or Mantel–Haenszel 95% confidence intervals (CI) for two binomial samples were calculated.

RESULTS

HPV infection frequency

The presence of HPV was detected for 13 of 38 cancer patients (34%). Of 31 cases selected for epigenetic analysis, 12 (39%) had

HPV infection. Most frequently HPV infection was detected in laryngeal (5 of 9; 56%), oropharyngeal (2 of 4) carcinomas and in carcinomas of cutis (3 of 5). Also, one case with lingual and one with hypopharyngeal carcinoma were infected by HPV.

After HPV typing, only HPV 16 was found in three tumour samples (one-by-one in oropharyngeal, hypopharyngeal and laryngeal carcinomas), and double infection with HPV 6 and 16 was found in laryngeal carcinoma. Other samples were negative for analysed HPV types (HPV 6, 11, 16 and 18).

In control group, 5 of 42 (12%) healthy volunteers were infected by HPV. However, only 11 samples were followed for evaluation of methylation status: 1 of these samples (9%) was infected with HPV. Interestingly, in this patient laryngeal papillomatosis was diagnosed. After typing, the HPV type was not specified.

Hypermethylation frequency in TSGs

In general, promoter hypermethylation in one or more of the TSGs was detected in 22 of 31 (71%) of the tumours under analysis. The prevalence of hypermethylation in the HNSCC was 23% for *p16,* 20% for *p14,* 19% for *RASSF1A,* 20% for *RAR*β, and 50% for *MGMT* genes. Promoter hypermethylation was also observed in oral brushings of cases without cancer. One case with *leucoplacia linguae* had hypermethylation in gene *p16*, one smoking healthy control in gene *MGMT*, and three cases in gene *p14*. One of the cases with hypermethylation in gene *p14* was a smoker, while two others did not declare their smoking behaviour.

Leukocyte DNA from healthy controls (n = 3) was negative for methylation in all genes. Representative results of MSP analysis are presented in Fig. 1.

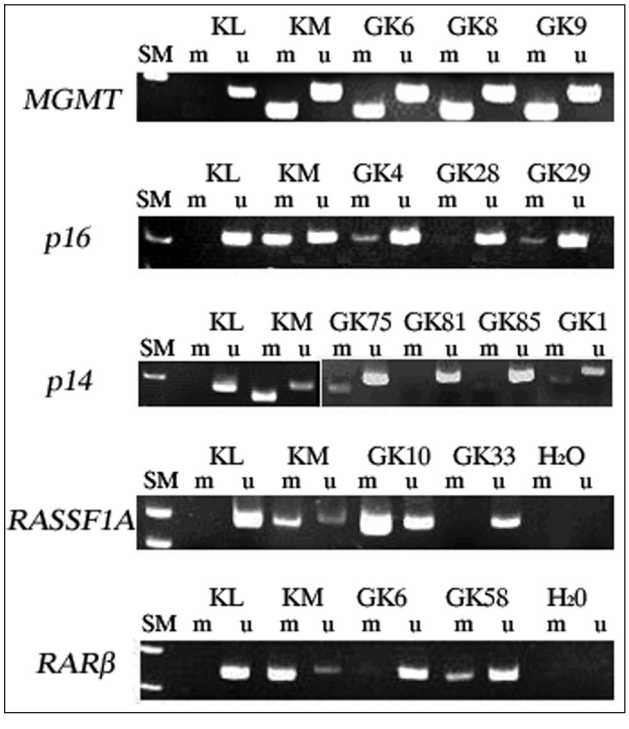

Fig. 1. Methylation pattern in the promoter regions of the *p16*, *p14*, *RARβ*, *RASSF1A*, and *MGMT* genes analysed by methylation specific PCR (MSP). m – methylated DNA, u – unmethylated DNA, GK – head and neck carcinoma, KL – leukocyte DNA from healthy control, KM – *in vitro* methylated DNA, H_20 – non-template (water) control

Hypermethylation and risk factors of HNSCC

The analysis of promoter hypermethylation of individual genes in relation to the risk factors and clinico-pathological characteristics of study group is shown in Table 2. Fisher's exact test did not reveal any significant differences in gene hypermethylation between HPV positive and negative cases. However, predominant hypermethylation of gene *RASSF1A* was observed in HPV positive cases as compared to negative ones (27% vs 8%), also the overall rate of hypermethylation (ORH; at least one gene hypermethylated) was slightly higher (69% vs 62%) in HPV positive cases.

In the study 30 cases were current smokers, 9 – never smokers and 3 did not declare smoking status. ORH was statistically significantly higher in smokers as compared to never smokers (73% vs 33%; p = 0.047). Also, genes *p16* and *p14* were more frequently hypermethylated in brushing specimens from smokers (Table 2). Hypermethylation was not detected among non-smoking controls, except one case with *leucolpacia liguae* who had gene *p16* hypermethylation. Smokers without cancer had higher ORH than never-smokers with or without cancer (Fig. 2). It is worth noting that gene *p16* methylation occurred in HNSCC from smokers only.

Hypermethylation and clinico-pathological features

A significant increase of TSGs hypermethylation was observed in HNSCC cases with a late stage (T3-T4) as compared to an early stage (T1-T2) cancer. Hypermethylation of at least one gene was detected in 84% of T3-T4 tumours and only in 50% of T1-T2 tumours (p = 0.056). Hypermethylation of genes *p16*, *p14* and *MGMT* was also predominant in late-stage tumours (Table 2).

Marked differences in the epigenetic profiles of HNSCCs of various localization were observed in our study. Hypermethylation of genes *p16* and *p14* was not detected in squamous cell carcinoma of cutis (Table 2). However, aberrant methylation of gene *RASSF1A* was more frequent in cutaneous tumours as compared to squamous cell carcinomas of larynx and hypopharynx (p = 0.08), or to other localizations, including oropharynx, lingua and sinus maxillae (p = 0.05). In contrast, hypermethyla-

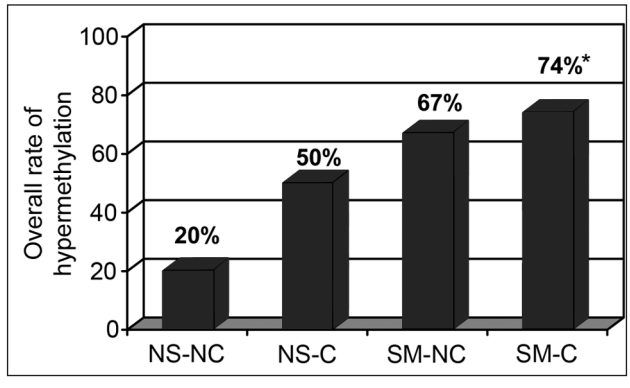

Fig 2. Overall rate of hypermethylation in smoking and non-smoking cases with and without head and neck cancer. NS-NC – never smokers without head and neck cancer; NS-C – never-smokers with cancer; SM-NC – smokers without cancer; SM-C – smokers with cancer; * statistically significantly (p < 0.05) higher than in never smokers without head and neck cancer

Table 2. **Association between methylation in genes *p16*, *p14*, *RASSF1A*, *RARβ* and *MGMT* and clinical-pathological variables of cases with head and neck tumours and controls**

Variables	n	Frequency of gene hypermethylation, %					ORM, %
		p16	*p14*	*RASSF1A*	*RARβ*	*MGMT*	
HPV status							
Negative	29	21	21	8	20	50	62
Positive	13	15	23	27	15	38	69
Smoking							
No	9	11	11	14	22	50	33
Yes	30	23	21	15	18	47	73*
Age							
<60	22	18	19	12	25	44	59
≥60	20	20	25	17	11	50	70
Gender							
Female	10	0	20	14	13	50	40
Male	32	25	23	14	20	44	72
Head and neck cancer							
No	11	9	27	0	0	25	45
Yes	31	23	20	19	20	50	71
Tumour stage							
T1-T2	12	8	0	27	27	33	50
T3-T4	19	32	32	13	21	58	84*[a]
Tumour localization							
Cutis	5	0	0	60	40	33	60
Larynx and hypopharynx	14	36	39	10	8	56	79
Other HNSCC	12	17	8	8*[b]	33	50	67

ORM – overall rate of hypermethylation (at least one of 5 genes methylated); HNSCC – head and neck squamous cell carcinoma; * statistically significant associations ($p \leq 0.05$; Fisher's exact test); *[a] borderline statistical significance ($p = 0.056$); *[b] compared to HNSCC of cutis.

tion of genes *p16* and *p14* was quite frequent in laryngeal and hypopharyngeal squamous cell carcinomas.

ORF and hypermethylation frequencies in individual genes were slightly higher in specimens obtained from older patients (≥60) as compared to younger ones (<60), but the differences were not statistically significant. Hypermethylation was also more prevalent in specimens collected from males than from females, but the female group was mainly composed of cases without HNSCC (7 of 10; 70%) and non-smokers (4 of 9; 44%).

DISCUSSION

In this study, we analysed the frequency of epigenetic alterations in HPV infection and smoking-related tumours of head and neck. In all the study population (38 cancer patients and 42 controls), HPV was detected in 13 cancer patients (34%) and in 5 healthy volunteers (12%). However, due to the insufficient quantity and quality of extracted DNA, not all samples were analysed for gene hypermethylation status. For the following analysis, samples from 36 cancer patients and 11 healthy volunteers were selected. In these samples, HPV infection was detected in 39% of HNSCCs, and 87% of the patients were smokers.

Our study revealed a significant effect of smoking on the induction of hypermethylation in TSGs involved in different pathways of cellular life. The overall rate of TSGs hypermethylation was higher in specimens from smokers, with or without head and neck cancer, thus showing a significant effect of smoking on head and neck pathogenesis. No significant associations were detected between TSGs hypermethylation and HPV infection. Genetic rather than epigenetic alterations might be caused by HPV infection, and this hypothesis remains to be examined in further studies.

The frequency of HPV infection detected in our study is similar to that reported in other studies on HNSCC. In many reports [20], the prevalence of HPV DNA ranges from 24% in the HNSCC of larynx to 46% in the HNSCC of the oral cavity and pharynx, with great discrepancies seen in different studies. The differences across studies in HPV detection rates of head and neck cancers may be related to the frequency of specific HNSCC sites evaluated, patient characteristics, and different assay methods used. Gillison et al. [21] found HPV 16 in 22%, HPV 18 in 0.4%, HPV 33 in 1.2%, and HPV 31 in 0.4% of 253 HNSCC patients. In our study, HPV 16 was found in three tumour samples, and a double infection with HPV 6 and 16 was found in one case. Other types of HPV were not detected in our study, probably due to the small number of cases analysed.

Cigarette smoking is the other important etiological factor causing HNSCC development. Tobacco smoke, referred as a chemical risk factor, contains well recognized carcinogens and pro-carcinogens such as benzo(a)pyrene (BAP), polycyclic aromatic hydrocarbon (PAH), arylamines and tobacco-specific nicotine-derived nitrosoamino ketone (NNK). Chronic exposure to tobacco carcinogens may induce DNA damages, leading to genetic and epigenetic alterations [22]. Significant associations between smoking-related parameters, such as smoking duration and pack-years, and hypermethylation of TSGs have been observed in carcinomas from smokers [reviewed in 23]. Hypermethylation of several TSGs have been observed in spu-

tum or bronchial brushes from cancer-free smokers, and the extent of gene hypermethylation in sputum from heavy smokers was shown to predict occurrence of cancer [23]. Associations between gene promoter methylation and tobacco carcinogen exposure were also shown in tobacco carcinogen-induced mouse and rat tumours [reviewed in 24]. In our study, high overall rates of hypermethylation were observed in smokers, either with head and neck cancer or without it. Smokers without HNSCC in our study had hypermethylation of genes *p14* and *MGMT*. Similarly, Rosas et al. [25] detected gene *p16* and *MGMT* promoter hypermethylation in saliva DNA from a healthy smoker, while no epigenetic changes were observed in saliva DNA from never smokers.

An essential association between tobacco carcinogen exposure and gene *p16* hypermethylation was observed in our study in cases with HNSCC. None of never-smokers with HNSCC had gene hypermethylation, while in smokers the frequency of gene inactivation reached 26%. Strong correlations between gene hypermethylation and smoking have been observed in lung cancer, another malignancy of respiratory tract [reviewed in 23]. Correlations were shown between gene *p16* hypermethylation in lung cancer and smoking duration, pack-years, smoking during adolescence and the time since quitting smoking [23]. Similarly, Hasegawa and colleagues [26] found a significant correlation of *p16* promoter hypermethylation and younger age at smoking initiation in HNSCC, supporting the notion that *p16* promoter methylation is an early event in the carcinogenesis of different tobacco-related tumours. Also, a link between *p16* and *p14* co-hypermethylation was established and correlated with tumour size, lymph node metastases, greater clinical stage in HNSCC [10, 12]. Co-hypermethylation of genes *p16* and *p14* was also observed in our study in three cases with a late T4 stage HNSCC.

A high overall rate of TSGs hypermethylation in HNSCC was observed in our study (71%). This is in line with other studies reporting gene hypermethylation rates from 47 to 100% [11, 27]. In our study, the most frequently altered gene was *MGMT* (50%), while the hypermethylation rate of other genes was about 20%. This is in agreement with other studies in HNSCC [8–12], where the frequencies of genes *MGMT, p16* for *RARβ* hypermethylation varied from 12 to 67%, and were slightly lower (from 0 to 30%) for genes *p14* and *RASSF1A*. Notably, the sampling of biomaterial by brushing of altered tissues, used in our study, had the same sensitivity as an analysis of surgical material conducted in other studies. It indicates that such a low-invasive procedure can be employed for the screening of high-risk groups, including heavy smokers, for the risk of HNSCC. Additional studies in bigger study groups of cancer patients and healthy volunteers adjusted for age, sex and smoking status are needed for a better understanding of the clinical importance of epigenetic biomarkers of head and neck cancer.

CONCLUSIONS

In summary, our study demonstrated a high incidence of smoking (87%) and HPV infection (39%) in HNSCC patients. Our results indicate that smoking is a strong factor for aberrant epigenetic events in upper airways and has a significant impact on head and neck carcinogenesis. Analysis of promoter hypermethylation of selected TSGs in biosamples obtained by low-invasive means can be a valuable biomarker for an early prediction of head and neck cancer risk in heavy smokers.

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NAVIKĄ SLOPINANČIŲ GENŲ PROMOTORIAUS HIPERMETILINIMAS GALVOS IR KAKLO NAVIKŲ LĄSTELĖSE

Santrauka

Galvos ir kaklo vėžys – tai viršutinių kvėpavimo takų piktybiniai navikai, dažniausiai atsirandantys dėl tabako ir alkoholio vartojimo bei žmogaus papilomos viruso (ŽPV) infekcijos. Ištyrę 38 ligonių ir 42 sveikų asmenų galvos ir kaklo srities navikų arba burnos epitelio nuograndas, ŽPV infekciją nustatėme 34% ligonių ir 12% sveikų asmenų mėginiuose. Promotorių DNR hipermetilinimas – tai naviką slopinančių genų inaktyvinimas, neretai nustatomas įvairių lokalizacijų navikuose. Norėdami įvertinti epigenetinių pažaidų svarbą galvos ir kaklo kancerogenezėje bei nustatyti ryšį su rūkymu ir ŽPV infekcija, tyrėme penkiuose naviką slopinančių genų hipermetilinimą ligonių bei sveikų asmenų mėginiuose. 71% navikų nustatytas bent vieno geno promotoriaus DNR hipermetilinimas. Epigenetinės pažaidos dažniau rastos rūkančiųjų epitelio nuograndose arba navikinėse ląstelėse ($p = 0,047$) nei nerūkančių asmenų mėginiuose. DNR metilinimo pakitimai buvo dažnesni vėlyvų stadijų navikuose. Mūsų tyrimas rodo, jog DNR metilinimo pažaidos yra svarbios galvos ir kaklo kancerogenezėje. Atrinktų genų promotorių hipermetilinimo analizė DNR, išskirtoje iš didelės rizikos asmenų burnos ertmės nuograndų, yra tinkama ankstyvos galvos ir kaklo navikų rizikos vertinimo priemonė.