VILNIUS UNIVERSITY

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DNA METHYLATION MARKERS OF LUNG AND HEAD-NECK TUMOURS

Summary of doctoral dissertation
Biomedical science, biology (01B)

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Doctoral dissertation has been done at Department of Botany and Genetics, Vilnius University in 2006 - 2010.

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Doctoral dissertation will be deffended at the public session of the Council of Biological Science at 12:00 pm on 22nd June, 2012 in the Great auditorium of the Faculty of Natural Sciences, Vilnius University.
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VILNIAUS UNIVERSITETAS

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INTRODUCTION

Lung and head-neck tumours are malignancies with high rates of incidences and deaths in Lithuania and world-wide. In the last decade, morbidity rates for lung and head-neck cancers remained stable with quite poor prognoses. For instance, 5-year prognoses for lung cancer patients are: 9% in Lithuania, 10% in Europe, and 15% in the world (Dobrovolskienė et al, 2010). The majority of cases with these tumours are active smokers or have been chronically exposed to second-hand tobacco smoke (SHS). Tobacco smoke contains a mixture of well known carcinogens and procarcinogens, which are shown to induce genetic and epigenetic changes (Hecht, 2003).

Recent studies suggest the association between promoter hypermethylation and tobacco smoke. Epigenetic alterations within promoter region of tumour suppressor genes (TSGs) are detectable in bronchial, oral epithelium and sputum of cancer-free smokers. Significant associations have been shown between promoter hypermethylation and smoking intensity or duration (Belinsky, 2004). Therefore epigenetic changes in TSG promoters become of diagnostic value (Esteller et al, 2001).

SHS has also been classified as a human carcinogen. It causes lung and head-neck tumours in neversmokers. Due to a small number of lung cancer cases in neversmokers and a lack of detailed tobacco smoke exposure data, genetic and epigenetic alterations in tumours from neversmoking patients with environmental tobacco smoke exposure are less examined. In order to determine the significance of epigenetic changes in pathogenesis of smoking (active or passive) related lung and head-neck tumours, we investigated DNA methylation changes in gene *p16*, *p14*, *RARB*, *RASSF1*, *MGMT* and *DAPK1* promoters.

Genes selected for the study are shown to be altered in lung and head-neck tumours. Their coding proteins play an important role in regulation of cell cycle, differentiation, cell division, DNA repair and apoptosis.

Aim of the study

The aim of this study was to determine the frequency and profile of epigenetic (DNA methylation) changes in lung and head-neck tumours; to determine the relationship between epigenetic changes and clinical, demographic factors, smoking behaviour or second-hand tobacco smoke exposure.

Tasks of research

1. To examine promoter hypermethylation in TSGs *p16*, *RARB*, *RASSF1*, *MGMT*, *DAPK1* in lung tumours, and to evaluate the association between aberrant promoter methylation and clinical, demographic characteristics and smoking behaviour.
2. To examine promoter hypermethylation in TSGs *p16*, *p14*, *RARB*, *RASSF1*, *MGMT* in scrapings of head-neck tumours and oral mucosa of patients without head-neck cancer, and to evaluate the association between aberrant promoter methylation and clinical, demographic characteristics and smoking behaviour.
3. To examine promoter hypermethylation of gene *MGMT* and MGMT protein expression in salivary gland carcinomas and histologically normal salivary gland tissues. To evaluate the association between promoter hypermethylation and protein expression

and clinical, demographic characteristics; to determine the association between promoter methylation and protein expression.

The statements being defended

1. Aberrant promoter methylation of TSGs in lung and head-neck tumours is a biomarker of tobacco smoke exposure (direct or indirect) and cancer.
2. DNA methylation markers can be analyzed in scrapings of oral epithelium and are suitable for an early prediction of head-neck cancer risk.
3. Promoter hypermethylation of gene *MGMT* causes the loss of MGMT protein expression in salivary gland carcinomas. This alteration is a significant predictor of outcome in salivary gland cancer.

Scientific novelty of the study

This study focused on cancer epigenetics. We analysed the pattern of promoter hypermethylation in non-small cell lung and head-neck tumours from neversmokers and smokers. Studies on promoter hypermethylation of TSGs in neversmokers with lung cancer are rare due to a low number of incidences (Kim et al, 2001, Jarmalaite et al, 2003). Data on lung cancer of current or former smokers is much more plentifull (Kim et al, 2001, Belinsky, 2004). In this study, we evaluated the association between DNA methylation changes and the effect of direct or indirect tobacco smoke in smokers and neversmokers, respectively. We showed that gene *p16* promoter hypermethylation is frequent in lung tumours from smokers and neversmokers with SHS exposure.

A lot of information on molecular alterations in head-neck carcinomas is available. However, due to variable localization of head-neck tumours, results are contradictory. In our study on head-neck tumours, gene *MGMT* promoter hypermethylation was the most informative DNA methylation marker. We showed that DNA methylation changes reflect harmful effect of tobacco smoke.

In this study, analysis of a large set of rare salivary gland carcinomas is presented. *MGMT* promoter hypermethylation and MGMT protein expression were analyzed in a homogenous group of head-neck tumours. Some data on protein expression in these tumours are published. However, epigenetic changes in these rare carcinomas and especially in histologically normal tissues are less investigated. Because of low number of salivary gland cancer cases in Lithuania, analysis on molecular alterations in these tumours are not examined in the country, to date. We showed that MGMT protein expression in salivary gland carcinomas correlates with clinical factors typical for patients with poor prognosis.

In this study, we aimed to supplement the fundamental knowledge on peculiarities of DNA methylation in lung and head-neck tumours. Also, the study is valuable for practical purposes since nowadays epigenetic markers are under establishment into cancer diagnostic systems.

Scientific and practical significance

Genetic and epigenetic changes in TSGs are caused by carcinogenic environmental exposure and have influence on pathogenesis of disease. Analysis of these molecular alterations provides information on the risk of cancer development, course of disease and sensitivity to treatment. Identification of novel biomarkers serves for development of curative strategies that focus on particular molecular targets. In our study, promoter

hypermethylation of TSGs *p16, p14, RARB, RASSF1, MGMT* and *DAPK1* was analyzed in lung and head-neck tumours. Our study on aberrant DNA methylation contributes to the development of novel molecular biomarkers that help in early detection of disease and evaluation of prognosis. In lung and salivary gland tumours, we determined the relationship between epigenetic changes, loss of protein expression and clinical, demographic factors. We showed that analysis of aberrant DNA methylation may help in prediction of cancer risk, while analysis of protein expression may serve in the assessment of prognosis for salivary gland cancer patients.

Analysis of epigenetic changes is already under establishment into diagnostics, especially in non-invasive diagnostics of various malignancies, preventive programmes for high risk subjects. In head-neck study, we showed that analysis of promoter hypermethylation of TSGs can be detected scrapings of tumour and oral mucosa, the analysis is informative, and methodological sensitivity is sufficient. It might be an additional tool to usual diagnostic methods.

Our study extends the knowledge on the significance of epigenetic changes in pathogenesis of lung, head-neck and salivary gland tumours. Along with this research, first studies on epigenetic changes in breast and prostate samples collected in Lithuania were carried out. Also, experience acquired in institutions abroad was applied in the laboratory in Lithuania.

Studies were carried out in Vilnius University, Department of Botany and Genetics, Finish Institution of Occupational Health, Department of Biological Mechanisms and Prevention of Work-Related Diseases and University of Erlangen-Nurnberg, Department of Experimental Tumour Pathology.

MATERIALS AND METHODS

The study groups and data collection

Lung cancer study. 212 of non-small cell lung cancer patients (period from August 1988 - October 1997) were included in this study. The cases were from the European multicentre study on non-smokers' lung cancer (n=78) and the study carried out in Finland on smokers' lung cancer (n=134) (Husgafvel-Pursiainen et al, 2000, Boffetta et al, 1998). Clinical, pathological characteristics and detailed data on tobacco smoke, including SHS exposure details for neversmokers, were obtained (Table 1).

Table 1. Demographic, clinical, pathological characteristics and distribution according to smoking status of lung cancer cases.

Parameters	**All cases, n (%)**	**Neversmokers, n (%)**	**Smokers, n (%)**
Age (mean ±SEM)	63.5±0.7	63.0±2.0	63.7±0.7
Sex			
Female	83 (39.2)	34 (72.3)	49 (29.7)
Male	129 (60.8)	13 (27.7)	116 (70.3)
Histology			
Adenocarcinoma	103 (48.6)	31 (66.0)	72 (43.6)
Squamous cell carcinoma	79 (37.3)	4 (8.5)	75 (45.5)

Other	30 (14.1)	12 (25.5)	18 (10.9)
Stage			
I-II	71 (67.0)	-	69 (66.3)
III-IV	35 (33.0)	-	35 (33.7)
SHS exposure*			
No	-	9 (21.4)	-
Yes	-	33 (78.6)	-
***TP53* gene**			
Wild-type	133(62.7)	44 (93.6)	89 (53.9)
Mutant	79 (37.3)	3 (6.4)	76 (46.1)
Total	**212**	**47**	**165**

* Data on SHS exposure not available for 5 neversmokers.

Head-neck cancer study. 31 of head and neck cancer patients from Institute of Oncology, Vilnius University (period from March 2006 - November 2006) were included in this study. As a control group, 11 volunteers with no cancer indication participated in the study. The main characteristics of the study group are presented in Table 2. Scrapings of tumour and oral mucosa were collected from cancer and control cases, respectively. Human papilloma virus (HPV) testing and typing was performed from the fresh material in the Institute of Oncology, Vilnius University.

Table 2. Demographic, clinical, pathological characteristics of head-neck cancer and control cases.

Parameters	**Cancer cases, n (%)**	**Control cases, n (%)**
Age, years		
<60	14 (45)	8 (72)
≥60	17 (55)	3 (27)
Sex		
Female	3 (10)	7 (64)
Male	28 (90)	4 (36)
Smoking status		
Neversmoker	4 (13)	5 (45)
Smoker	27 (87)	3 (27)
HPV		
No	19 (61)	10 (91)
Yes	12 (39)	1 (9)
Stage		
I-II	12 (39)	-
III-IV	19 (61)	-
Localization		
Cutis of head-neck region	5 (16)	-
Pharynx, larynx	14 (45)	-
Other	12 (39)	-
Total	**31**	**11**

Salivary gland cancer study. 287 of salivary gland cancer patients who underwent surgical resection at Department of Otorhinolaryngology, University Hospital of

Erlangen, Department of Otorhinolaryngology and Maxillofacial Surgery, Nuremberg City Hospital, and Department of Otorhinolaryngology, Regensburg University Hospital between February 1988 - July 2008 were included in this study. Removed tumours and histologically normal salivary gland tissues were collected and Hematoxylin & Eosin stained slides were evaluated. The main characteristics of the study group are presented in Table 3.

Table 3. Demographic, clinical and pathological characteristics of salivary gland carcinoma cases.

Parameters	Cases, n (%)
Age, years	
<60	131 (45.8)
≥60	155 (54.2)
Sex	
Female	150 (52.4)
Male	136 (47.6)
Tumour localization	
Parotid glands	200 (69.9)
Submandibular glands	42 (14.7)
Minor salivary glands	43 (15.0)
Sublingual glands	1 (0.4)
Histology	
Acinic cell carcinoma	41 (15.3)
Adenoid cystic carcinoma	46 (17.2)
Mucoepidermoid carcinoma	42 (15.7)
Squamous cell carcinoma, Adenocarcinoma, not otherwise specified, Salivary duct carcinoma	101 (37.7)
Myoepithelial carcinoma, Malignant mixed tumour	21 (7.8)
Basal cell adenocarcinoma, Epithelial-myoepithelial carcinoma, Polymorphous low-grade adenocarcinoma	17 (6.3)
Disease stage	
I	66 (23.3)
II	62 (21.9)
III	62 (21.9)
IV	93 (32.9)
Grade	
I	88 (30.9)
II	68 (23.8)
III	129 (45.3)
pT	
1	76 (31.9)
2	94 (39.5)
3	68 (28.6)

Metastases	
No	258 (91.8)
Yes	23 (8.2)
Node involvement	
No	190 (70.4)
Yes	80 (29.6)
Death	
No	178 (62.2)
Yes	108 (37.8)
Total	**287**

Control DNA. The following DNAs from cell lines with known methylation profiles were used in all experiments: T24 (ATCC No. HTB-4), H157 (ATCC No. CRL-5802) and DLD1 (ATCC No. CCL-221) (all obtained from American Type Culture Collection). Leukocyte DNA from healthy controls and human placenta DNA (Deoxyribonucleic acid from human placenta, Millipore) were used as negative controls. *In vitro* methylated leukocyte DNA (using bacterial SssI methylase) or commercially available methylated human placenta DNA (CpGenome universal methylated DNA, Sigma) served as positive controls.

Analysis of promoter methylation

DNA extraction. Genomic DNA from fresh frozen lung cancer samples was isolated using standard proteinase-K digestion and phenol-chloroform-isoamyl alcohol purification followed by ethanol precipitation. Formalin-fixed paraffin-embedded lung cancer samples were dewaxed with xylene prior to proteinase-K digestion. DNAs from tumour scrapes of head-neck cancer patients and oral mucosa scrapes of control cases were extracted using the column method according to the manufacturer's protocol (SorpoClean TM Genomic DNA Extracion Module, SORPO Diagnostics). DNAs from salivary gland cancer cases were extracted from formalin-fixed paraffin-embedded samples using Nucleospin® Tissue kit (Macherey-Nagel) in accordance with manufacturer's protocol.

Bisulfite treatment. For lung cancer samples, 1 µg of genomic DNA was denatured with 3 M NaOH for 15 min at 37°C, and then was exposed to bisulphite modification with 2.3 M sodium metabisulphite and 10 mM hydroquinone (all from Sigma-Aldrich Inc) for 14-16 hr at 50°C. Modified DNA was purified with the Wizard DNA Clean-up System (Promega) and desulphonated with 3 M NaOH treatment for 15 min in 37 °C. DNA was precipitated with 70% ethanol and dissolved in 40 µl of sterile water.

Head-neck cancer samples were treated with bisulfite using protocol described above, except of lower DNA input. Salivary gland cancer samples were treated with bisulfite using CpGenome DNA modification kit (Millipore) in accordance with the manufacturer's protocol.

Methylation specific PCR. DNA methylation in lung and head-neck samples was studied by methylation specific PCR (MSP). MSP was set up as described earlier

(Herman et al, 1996). PCR primers specific for methylated (M) and unmethylated (U) sequences within the 5' region of the *p16*, *p14*, *RARB*, *RASSF1*, *MGMT*, and *DAPK1* genes were selected from publications (Herman et al, 1996, Esteller et al, 2000, Virmani et al, 2000, Burbee et al, 2001, Esteller et al, 1999, Katzenellenborgen et al, 1999). The PCR mixture of 50 µl contained 1-2 µl of modified DNA template, 1xPCR buffer, 0.4 mM of each deoxynucleotide triphosphate, 2.5 mM of $MgCl_2$, 1% of dimethylsulfoxide, forward and reverse primers at the final concentration of 6 ng/µl and 1.25 U of AmpliTaq Gold polymerase (Applied Biosystems). PCR conditions were: on average, 35-39 cycles of amplification and 60-66 °C annealing temperature was used. PCR products were analysed under UV light on ethidium bromide stained non-denaturing polyacrylamide gel. Gene hypermethylation was considered present when MSP products were detected in reactions with the primers for M and U sequences, or M sequence, only.

Quantification of DNA methylation by pyrosequencing. DNA methylation in salivary gland samples was studied by pyrosequencing method. Pyrosequencing followed bisulfate treatment and PCR. Primers from commercially available kit (Biotage AB) were used to amplify +17-39 bases in the promoter region of *MGMT* gene. PCR was set up as described by manufacturer. For pyrosequencing, biotin labeled PCR product was immobilized onto Streptavidin Sepharose High Performance beads (Biotage AB), purified, washed, and denaturated. Biotinylated strand was annealed to sequencing primer (0.3 µM final concentration) and was subjected to sequencing analysis on the PyroMark Q24 instrument (Biotage AB) with PyroGold reagents according to the manufacturer's protocol. The results were analyzed by PyroQ-CpG 1.0.10 software (Biotage AB). The methylation level was recorded as relative CT ratio (%). Average methylation level was calculated from methylation percentages for each CpG analyzed in the sample. 10% cut-off (methylation level at 5 CpGs <10% *versus* ≥10%) was used as a threshold for describing the methylation status of a sample.

Immunohistochemical evaluation of protein expression. Tissue microarrays from formalin-fixed paraffin-embedded salivary gland tumour and histologically normal tissue specimens (punch diameter 2mm) were constructed. Protein expression analysis was performed on 3-µm thin sections using standard Hematoxylin & Eosin staining protocol. Primary mouse monoclonal antibody SPM287 (Abcam) and the secondary antibody Envision+ (Dako) were used. Nuclear staining was considered. Protein expression was quantified manually at 200x magnification and scored as absent or low staining (<30% of cells stained), and present (≥30% cells stained).

Statistical analysis. The data on hypermethylation for each gene was summarized as a methylation frequency (%). The methylation index (MI, number of genes methylated divided by the number of genes examined) was calculated. Logistic regression analysis, both univariate (unadjusted) and multivariate (adjusted) was used to estimate odds ratios (OR) and 95% confidence intervals for hypermethylation. Student's t-test was used for comparison of continuous variables. SAS/STAT software (SAS Institute) version 9.2 was used. A p-value <0.05 was considered statistically significant.

RESULTS

Promoter hypermethylation of TSGs *p16, RARB, RASSF1, MGMT* and *DAPK1* in lung tumours

Promoter methylation and clinical, pathological and demographic features. Promoter methylation was frequent in lung tumours, with 71% (150/212) of the cases showing methylation in one or more of the genes. The overall mean MI was 0.23. The prevalence of methylation in lung tumours was 20.8% for *p16*, 31.1% for *RARB*, 28.9% for *RASSF1*, 15.1% for *MGMT* and 19.8% for *DAPK1* (Figure 1; Supplementary table 1).

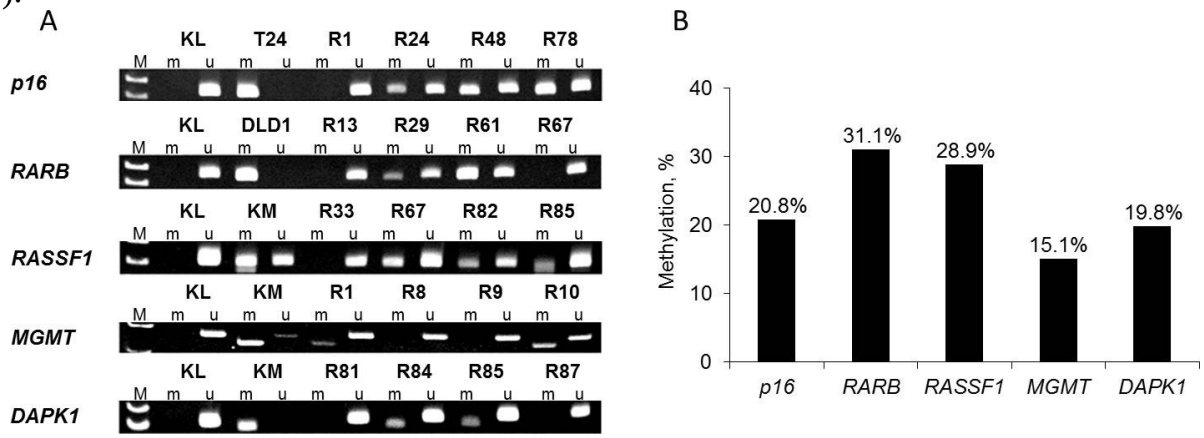

Figure 1. The pattern of promoter methylation of genes *p16, RARB, RASSF1, MGMT* and *DAPK* (A). The frequencies of promoter methylation in lung tumours (B). M - molecular marker, KL - leukocyte DNA from healthy control, KM - *in vitro* methylated DNA, T24 - cell line of human bladder cancer, R - lung cancer cases, m - methylated DNA, u - unmethylated DNA.

Aberrant methylation of *p16* was more common in males than females (27.9% *versus* 9.6%; p=0.002; Figure 2), while methylation of *RARB* promoter was predominant in female patients (39.8% *versus* 25.6%; p=0.044). The *DAPK1* gene also tended to be more frequently methylated in tumours from female patients (p=0.076). In multivariate analysis, the association between promoter methylation of gene *p16* and sex remained statistically significant (p=0.018).

With regard to tumour histology, *p16* promoter methylation occurred in 34.2% of squamous cell carcinomas, and it was significantly more prevalent than in adenocarcinomas (p=0.001; Figure 2). *RARB* methylation occurred less frequently in tumours of other histologies than in adenocarcinomas (p=0.012). In multivariate analysis, both associations showed statistical significance (p=0.025 for *p16*; p=0.006 for *RARB*).

The other characteristics (age at diagnosis, disease stage) were not significantly associated with the promoter methylation in any of the genes studied.

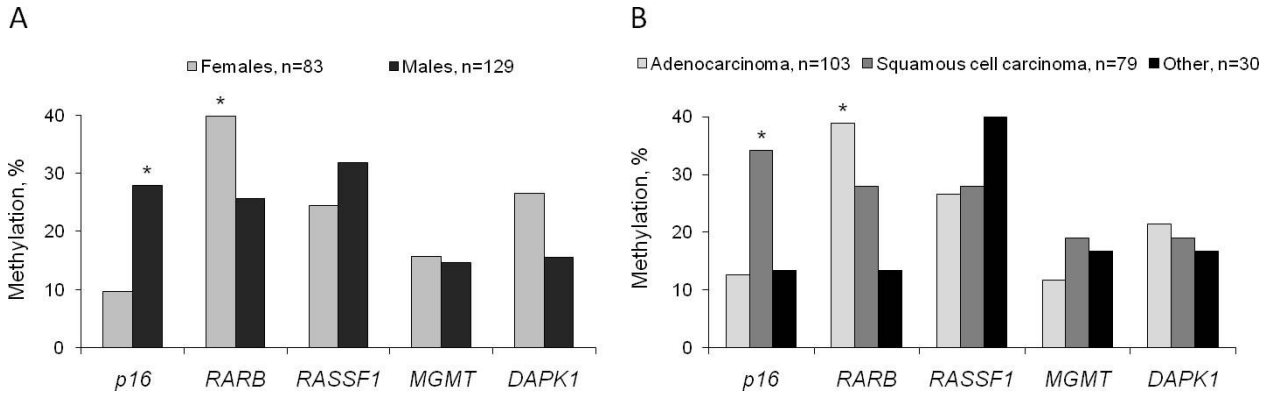

Figure 2. Frequencies of promoter methylation according to sex (A), and tumour histology (B). * - statistically significant differences.

Aberrant methylation and smoking. The observed frequencies of hypermethylation of five TSGs were at higher or comparable level in lung tumours from smokers compared to neversmokers (Figure 3; Supplementary table 1). Methylation of *p16* was predominant in smokers with borderline significance (p=0.066). In this group, former smokers had a statistically significant 2-fold higher risk of *p16* hypermethylation as compared to current smokers (OR 2.27; 95% CI 1.02-5.08; Figure 4). The risk was even more pronounced (OR 4.25; 95% CI 1.36-16.06; p=0.009), when former smokers were compared to the group of neversmokers. In contrast, *RARB* hypermethylation was less commonly observed in lung tumours from smokers (27.9%) as compared to neversmokers (42.6%). Neither intensity (cigarettes per day, pack-years) or duration of smoking, nor age at starting smoking were associated with TSG hypermethylation in smokers.

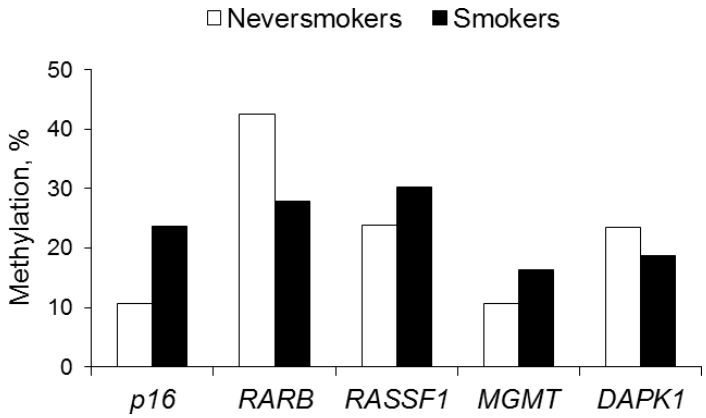

Figure 3. Frequencies of promoter methylation in lung tumours according to smoking status.

Aberrant methylation and SHS exposure. When the effect of SHS was considered, the mean MI in the tumours of SHS-exposed neversmokers was close to MI seen in smokers (Figure 4). MI was elevated, although non-significantly, in neversmokers with SHS exposure, as compared to neversmokers without such exposure (0.24 *versus* 0.16). Similarly, the number of affected genes was higher in smokers and in the SHS-exposed neversmokers as compared to the non-exposed neversmokers. Fisher test revealed statistically significant differences of *p16* hypermethylation: the highest odds were seen when former smokers were compared to neversmokers not exposed to

SHS (OR 6.104; 95%CI 0.89 - ∞; Figure 4). *RARB* methylation was frequent in the overall group of neversmokers, and it predominantly occurred in tumours from SHS-exposed cases (48.5% *versus* 22.2% in non-exposed to SHS patients). *DAPK1* gene also showed the prevalence of hypermethylation in SHS-exposed neversmokers (30.3% *versus* 11.1 % in non-exposed to SHS patients).

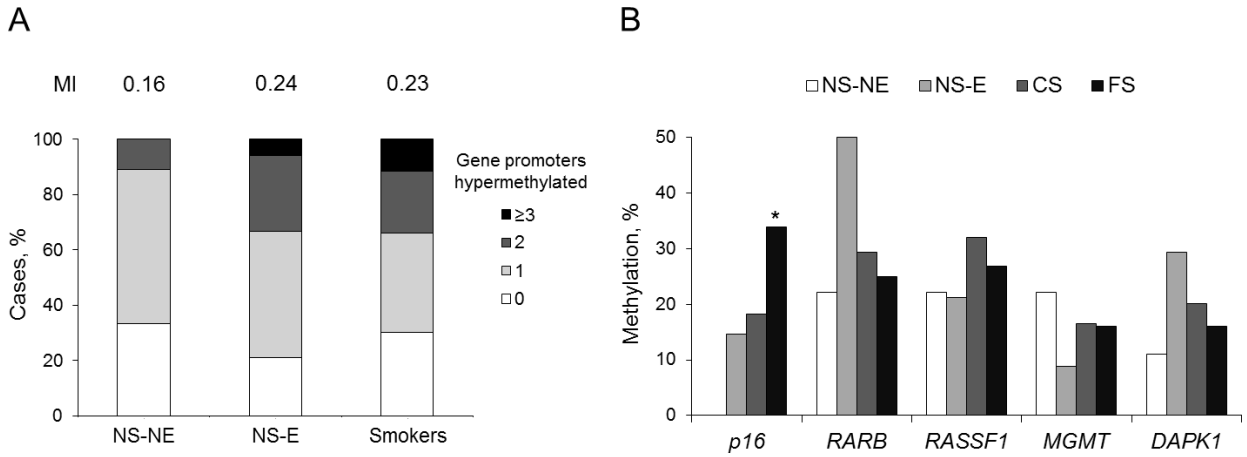

Figure 4. Proportion of cases (%) with 0, 1, 2, and ≥3 genes hypermethylated, among smokers and neversmokers (A). Frequencies of promoter methylation in lung tumours according to smoking and second-hand tobacco smoke exposure status (B). NS-NE - neversmokers non-exposed to SHS, NS-E - neversmokers exposed to SHS, CS - current smokers, FS - former smokers. * - statistically significant differences.

Aberrant methylation and *TP53* mutation. The same series of lung cancers were previously analyzed for *TP53* gene mutations (Husgafvel-Pursiainen et al, 1999, Husgafvel-Pursiainen et al, 2000). Mutations in gene *TP53* were detected in 46.1% (76/165) of smokers and 6.4% (3/47) of neversmokers. The mean MI was significantly higher in tumours with *TP53* mutation as compared to the cases with a wild-type gene (0.27 *versus* 0.21; p=0.018; Figure 5). Of the individual genes, hypermethylation of *p16* and *MGMT* genes was more common in *TP53*-mutated lung tumours (p=0.034 and p=0.072, respectively). In multivariate analysis, the association between the mutation status of gene *TP53* and methylation in ≥1 gene promoters remained statistically significant (p=0.027).

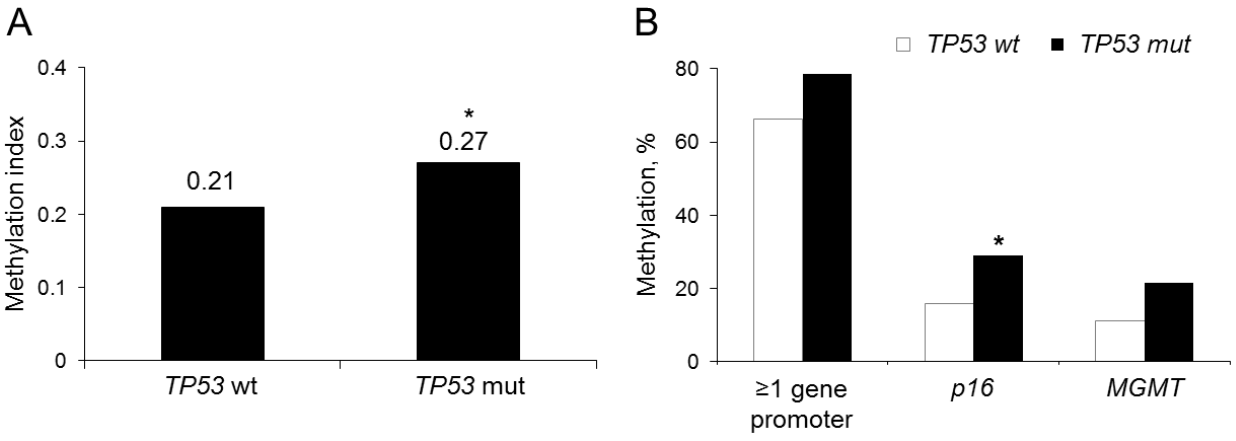

Figure 5. Methylation index in lung tumours with a wild-type *TP53* gene and mutated *TP53* gene (A). The frequencies of promoter hypermethylation in ≥1 gene and genes *p16* and *MGMT* in lung tumours (B). * - statistically significant differences.

Promoter hypermethylation of TSGs *p16, p14, RARB, RASSF1* and *MGMT* in head-neck tumours

Promoter methylation and clinical, pathological and demographic features. Promoter methylation was frequent in head-neck tumours, with 71% (22/31) showing methylation in one or more of the genes. The prevalence of hypermethylation in tumours was 23% for *p16*, 20% for *p14*, 20% for *RARB*, 19% for *RASSF1*, and 50% for *MGMT* (Figure 6; Supplementary table 2). Promoter methylation was also observed in oral scrapings of 5 cases without cancer (n=11). One case with *leucoplacia linguae* had hypermethylation in gene *p16*, one smoking healthy control - in gene *MGMT*, and 3 cases - in gene *p14*. One of the cases with hypermethylation in gene *p14* was smoker, while two others did not declare their smoking behaviour.

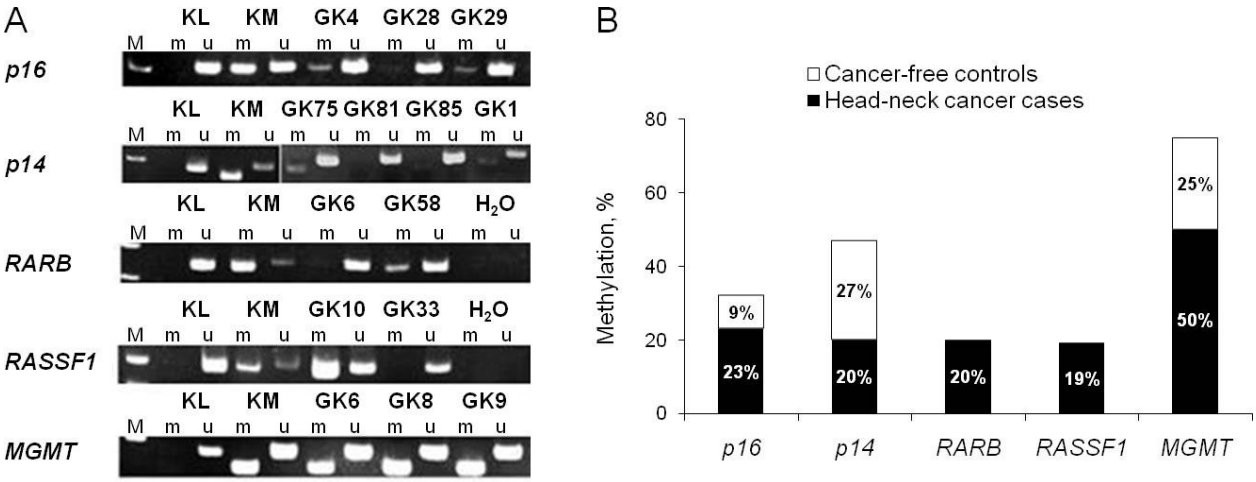

Figure 6. The pattern of promoter hypermethylation of genes *p16*, *p14*, *RARB*, *RASSF1* and *MGMT* (A). The frequencies of promoter hypermethylation in head-neck cancer cases and cancer-free controls (B). M - molecular marker, KL - leukocyte DNA from healthy control, KM - *in vitro* methylated DNA, m - methylated DNA, u - unmethylated DNA, GK - head-neck carcinoma, H_2O - non-template (water) control.

No significant differences in gene hypermethylation were detected between HPV positive and negative cases in the whole study group. However, predominant methylation of gene *RASSF1* was observed in HPV positive cases as compared to negative ones (27% *versus* 8%). Also, the overall rate of methylation (ORH; at least one gene hypermethylated) was slightly higher in HPV positive cases (69% *versus* 62%).

When the clinical factors were considered, the ORH was found markedly increased in late stage (T3-T4) tumours as compared to early stage (T1-T2) tumours (84% *versus* 50%; p=0.056; Figure 7). In late stage tumours hypermethylation of genes *p16*, *p14* and *MGMT* was predominant.

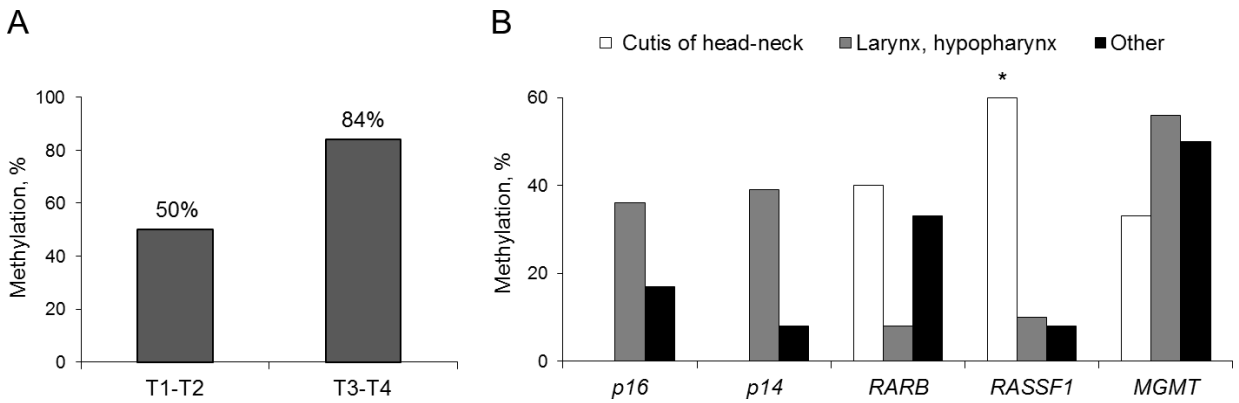

Figure 7. Overall rate of hypermethylation in early (T1-T2) and late (T3-T4) stage head-neck carcinomas (A). The frequencies of promoter hypermethylation in tumours of various head-neck localizations (B). * - statistically significant differences.

Marked differences in hypermethylation profiles were observed in tumours of various localizations (Figure 7). Hypermethylation of genes *p16* and *p14* was not detected in squamous cell carcinomas of cutis. However, aberrant methylation of gene *RASSF1* was more frequent in cutaneous tumours, as compared to tumours of larynx and hypopharynx (p=0.08), or to other localizations, including oropharynx, lingua and sinus maxillae (p=0.05). In contrast, hypermethylation of genes *p16* and *p14* was quite frequent in laryngeal and hypopharyngeal carcinomas.

ORH and hypermethylation frequencies in individual genes were slightly higher in specimens obtained from older patients as compared to younger ones (≥60 versus <60 years, whole study group), but the differences were not statistically significant. Hypermethylation was also more prevalent in specimens collected from males than females (whole study group), but female group mainly consisted of cases without head-neck cancer (7/10; 70%) and of neversmokers (4/9; 44%).

Aberrant methylation and smoking. In the whole study group (n=42), 30 cases were smokers, 9 - never smokers and 3 did not declare smoking status. ORH was significantly higher in smokers as compared to neversmokers (73% *versus* 33%; p=0.047; Figure 8). Genes *p16* and *p14* were more frequently hypermethylated in brushing specimens from smokers. Hypermethylation was not detected among non-smoking controls, except one case with *leucoplacia linguae* who had gene *p16* hypermethylated. Smokers without cancer had higher ORH, than neversmokers with or without cancer (Figure 8). Statistically significant differencies (p<0.05) were observed between smokers with head-neck cancer and neversmokers without cancer.

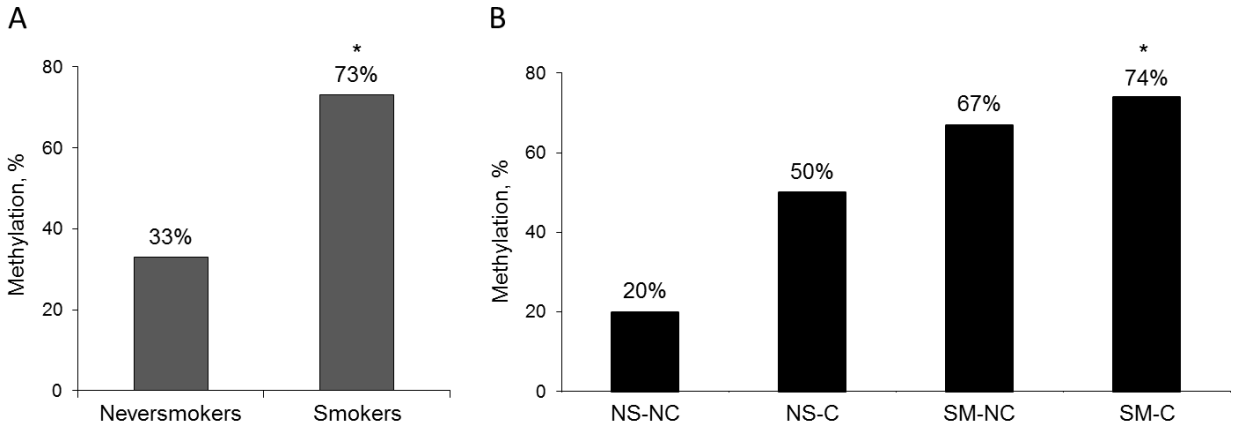

Figure 8. Overall rate of hypermethylation in neversmokers and smokers (A). Overall rate of hypermethylation in smoking and neversmoking head-neck cancer cases and cancer-free controls (B). NS-NC - neversmokers without head-neck cancer, NS-C - neversmokers with cancer, SM-NC - smokers without head-neck cancer, SM-C - smokers with cancer. * - statistically significant differences.

Promoter hypermethylation of TSG *MGMT* and loss of MGMT protein expression in salivary gland tumours

Promoter methylation of gene *MGMT* and clinical, pathological and demographic features. Aberrant promoter methylation (average methylation level at 5 CpGs ≥10%) was detected in 27.8% (10/36) of tumour specimens (Figure 9) and in 15.8% (3/19) of histologically normal salivary gland tissues. Promoter hypermethylation in normal salivary gland tissues was detected only in case of methylation in corresponding tumour. The average methylation level of *MGMT* in tumours was increased in comparison to histologically normal tissues (2-35% *versus* 1-10%, respectively; Figure 9).

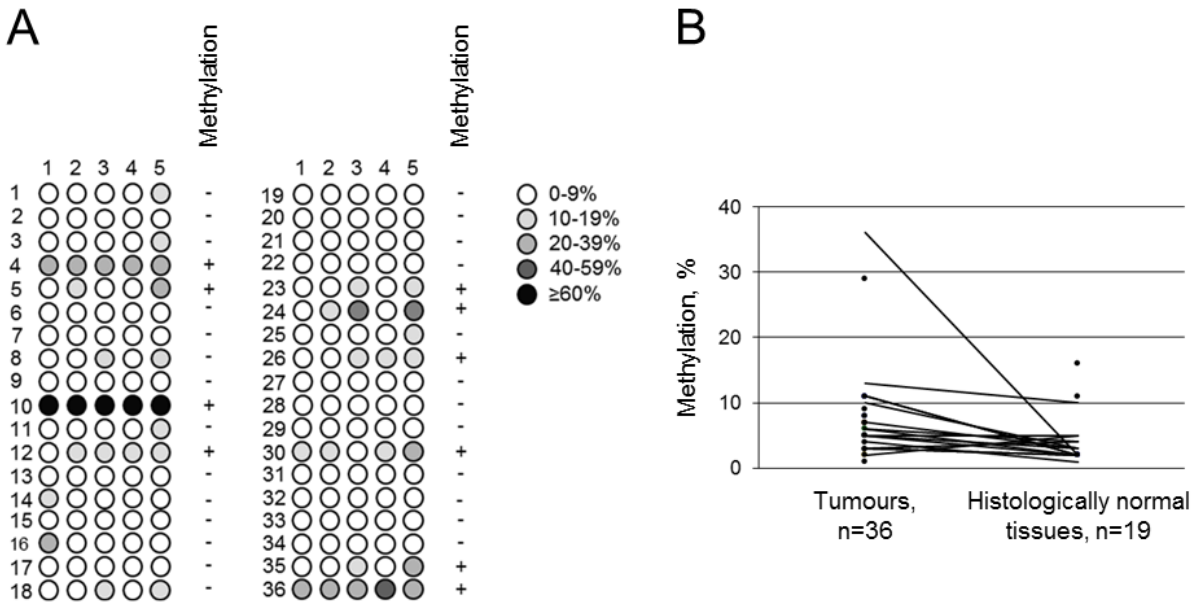

Figure 9. Methylation profile of an individual CpG sites in *MGMT* gene promoter in salivary gland carcinomas (1-36) (A). Each circle represents one CpG in a panel of 5 studied. Sample is methylated (+) when the mean methylation value is ≥10%. The

average methylation level of *MGMT* in tumours and histologically normal tissues (B). Results are represented in tumour - non-tumour pairs (straight) and non-paired samples (dots).

The methylation status of *MGMT* in tumours did not correlate with any clinical, pathological and demographic variables. However, the statistically significant (p=0.021) correlation was observed between *MGMT* promoter hypermethylation and loss of MGMT protein expression (Figure 10).

Loss of MGMT protein expression. The protein loss was detected in 39.0% (112/287) of salivary gland tumours. When tumour - histologically normal tissue pairs (n=70) were considered, the MGMT protein loss was observed at higher frequency in salivary gland tumours (p=0.001; Figure 10).

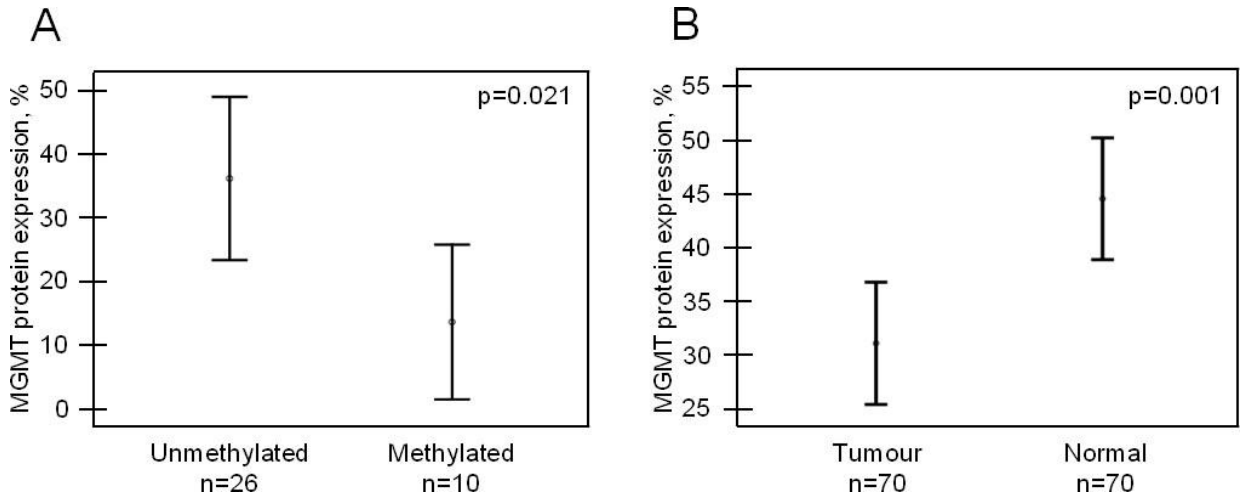

Figure 10. The association between *MGMT* promoter hypermethylation and MGMT protein expression in salivary gland tumours (A). The protein expression detected in tumours and matched histologically normal tissues (B).

Significant associations were observed between loss of MGMT protein and histological type, grade and lymph node involvement (Supplementary table 3). With regard to demographic parameters, MGMT loss strongly correlated with age of the patients and sex. No associations were observed with tumour localization, stage, size of the tumours and distant metastases.

Patients having tumours with MGMT protein loss had a worse prognosis than those showing MGMT protein expression (Figure 11). 5-year overall survival in these two groups with and without MGMT loss was 55.9% and 69.9%, respectively. The 10-year survival magnified the prognostic difference (38.0% *versus* 59.9% in groups with MGMT loss and without, respectively). There was a high predictive value for MGMT loss on the clinical outcome of these patients (p=0.004). In an univariate Cox regression analysis, patients having tumours with MGMT protein loss showed a 1.7-fold risk of dying of disease. However, in the multivariate analysis, MGMT loss did not retain an independent prognostic value.

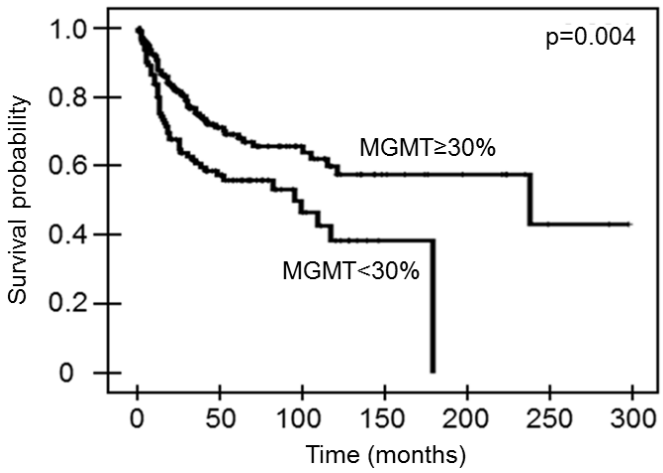

Figure 11. Kaplan-Meier curve for survival of patients with salivary gland carcinoma.

DISCUSSION

Our study aimed at better understanding of the association between tobacco smoke and promoter hypermethylation in respiratory tract tumours. We focused our methylation analyses on TSGs *p16*, *p14*, *RARB*, *RASSF1*, *MGMT* and *DAPK* in 212 of lung tumours, 31 of head-neck tumours and 36 of salivary gland carcinomas. In addition, 11 histologically normal head-neck and 19 salivary gland tissues were examined.

Our data show that epigenetic alterations are frequent in lung and head-neck tumours. Promoter hypermethylation in ≥1 gene occurred in 70% cases. Similarly, high frequencies of hypermethylation have been observed in lung (79 - 92%) and head-neck tumours (42 - 100%) by other authors (Cirrincione et al, 2005, Ulivi et al, 2006, Steinmann et al, 2009, Calmon et al, 2007, Kishi et al, 2005, Paluszczak et al, 2011). None of the individual genes analyzed showed exclusively high prevalence in lung tumours. In comparison, in head-neck carcinomas, *MGMT* gene was frequently hypermethylated. This is well in line with the literature data (Steinmann et al, 2009, Paluszczak et al, 2011). In our study, detailed comparison of findings in lung and head-neck carcinomas was derogated because of incomparable number of patients, different origin (whole tumour tissues, tumour scrapings) and quality of study material (fresh frozen, paraffin embedded tumour tissues). Also, the sensitivity of methods used and evaluation of results (quantitative, qualitative) differed.

We analyzed the prevalence and pattern of TSG promoter methylation in smoking related malignancies. Tobacco smoke is considered a chemical risk factor which contains over 60 carcinogens and procarcinogens. Chronic exposure to the complex mixture of tobacco smoke constituents induce genetic and epigenetic alterations that may, in turn, lead to malignant growth and cancer development. In this study, we compared the frequency of epigenetic changes in lung and head-neck tumours obtained from smokers and neversmokers. In lung cancer, hypermethylation within gene *p16* promoter was more prevalent in smokers than neversmokers. In the whole group of smokers, hypermethylation of *p16* was predominant in former smoking patients. This finding is in line with other studies on lung tumours from smokers and with the previous study of our research group (Belinsky et al, 1998, Kim et al, 2001, Toyooka et al, 2004, Jarmalaite et al, 2003). In our study, the association between tobacco smoke and *p16*

hypermethylation was also observed in head-neck tissues. None of neversmoking cancer patients had alterations in this gene, while 26% of active smokers with head-neck cancer had gene altered.

In our study, no significant associations between hypermethylation and smoking habits (duration, intensity, age at start smoking) were observed in lung tumours from active smokers. However such associations were shown by other authors in cases with lung (Kim et al, 2001, Jin et al, 2010, Vineis et al, 2005) and head-neck cancer (Hasegawa et al, 2002).

In head-neck tumours, we found the association between aberrant *p16* methylation and smoking behaviour. In neversmokers with head-neck cancer, promoter hypermethylation of *p16* was not detected, while methylation in this gene was observed in 26% of smokers. Overall rate of hypermethylation in smokers (with or without head-neck cancer) was increased when compared to neversmokers. In smoking cases without head-neck cancer, *p14* and *MGMT* promoters were hypermethylated. Similar findings were published previously. Alterations of *CDKN2* locus (*p16*) and *MGMT* were shown in saliva DNA from healthy smokers (Rosas et al, 2001). However, in contrast to our data, methylation changes were not detected in neversmokers. In addition, MGMT expression was analyzed in series of normal oral mucosa, dysplasia and early oral squamous cell carcinoma (Rodriguez et al, 2007). The loss of MGMT protein expression was observed in carcinomas from smokers. High frequencies of hypermethylation were shown in histologically normal tissues of head-neck cancer patients (Paluszczak et al, 2011). Interestingly, in this study, all subjects were alcohol drinkers and/or smokers.

No uniform notion on the importance of epigenetic changes in tobacco-related carcinogenesis exists. Higher frequencies of methylation and methylation index have been detected in smokers than neversmokers (Toyooka et al, 2003, Toyooka et al, 2006, Liu et al, 2007). Also, promoter hypermethylation of several TSGs have been detected in sputum and bronchial lavage DNA from healthy smokers. Moreover, the number of genes hypermethylated in chronic smokers was reported to inform about tumour development (Vineis et al, 2005). Discrepant results have been provided by other studies (Pulling et al, 2003, Divine et al, 2005), mainly showing similar prevalence of hypermethylation in smokers and neversmokers. These inconsistences may arise from differences in smoking intensity or the lack of detailed and systematic data on SHS exposure for neversmokers.

In our study, neversmokers with lung cancer had an extensive data on carcinogenic exposure to SHS at home or at work (Boffetta et al, 1998, Husgafvel-Pursiainen et al, 2000). We observed the absence of epigenetic changes in gene *p16* and low methylation index in neversmokers without SHS exposure. In contrast, exposed to SHS neversmoking subjects had similar methylation index as active smokers. Thus, gene *p16* methylation was detected in tumours from tobacco smoke affected (smokers and neversmokers) subjects, only. We showed that molecular alterations in lung carcinomas from smokers (active or passive) and neversmokers differ. Similarly, *RARB* methylation was twice frequent in tumours from SHS exposed than unexposed lung cancer patients. This might reflect the carcinogenic effect of SHS.

Aberrant methylation of TSG promoters are informative molecular markers in tobacco smoke-related tumours. Methylation of gene *p16* has been detected in sputum, plasma and bronchial lavage DNA from cancer free smokers (Belinsky et al, 2006, Baryshnikova et al, 2008, Cirincione et al, 2006, Destro et al, 2004, Kersting et al, 2000)

and is debated as a tool for early lung cancer detection, since this epigenetic change precedes development of malignant disease. In our study, promoter methylation of *p16* was observed in lung carcinomas from smokers and SHS exposed neversmokers, and in head-neck tumours from smokers. In lung and head-neck tumours from neversmokers (active or passive), aberrant methylation of *p16* was not detected. Thus, we showed that changes in DNA methylation within TSG promoters reflect the harmful effect of tobacco smoke and SHS exposure.

The same series of lung cancers were previously analyzed for *TP53* gene mutations (Husgafvel-Pursiainen et al, 1999, Husgafvel-Pursiainen et al, 2000). We found that smokers exhibited increased risk of *TP53* mutation as compared to neversmokers. Further, the exposure to SHS doubled the risk of *TP53* mutation in neversmokers. The present study revealed the association between occurrence of *TP53* mutation and hypermethylation of *p16* and *MGMT* genes. Epigenetic inactivation of *MGMT* increases genetic instability of cells, since the gene encodes a DNA repair enzyme responsible for removal of alkyl groups from the O^6 position of guanine. As reported, the inactivation of *MGMT* in lung tumours is associated with increased occurrence of *TP53* mutations, especially G:C to A:T transitions (Wu et al, 2008). These mutations are shown to be frequent in lung tumours from neversmoking patients (Husgafvel-Pursiainen et al, 2000), and possibly show the effect of SHS for the loss of genetic stability.

Smoking and human papilloma virus are known to be involved in pathogenesis of head-neck tumours. No significant associations were detected between TSG hypermethylation and HPV infection in our study. Genetic rather than epigenetic alterations might be caused by HPV infection, and this hypothesis remains to be examined in further studies. Data on this relationship is poor. Associations are shown with less known genes (Bennet et al, 2010) or negative correlations between promoter hypermethylation and HPV infection are presented (Dong et al, 2003).

In our study, we evaluated the association between epigenetic changes and clinical, demographic parameters. Hypermethylation of particular genes was associated with gender and tumour histology in lung cancer study. Hypermethylation of *p16* was predominant in squamous cell carcinomas and in tumours from men. Conversely, we observed *RARB* hypermethylation to be more frequent in tumours from females, and in adenocarcinomas. Our results are in line with the majority of previous studies on resected tumour tissues, in which *p16* hypermethylation was observed in 24-56% of squamous cell carcinomas and to a lesser extent in other histologies (Grote et al, 2005, Kim et al, 2001). Hypermethylated *RARB* promoter typically predominates in adenocarcinomas from female patients, usually neversmokers (Toyooka et al, 2003, Grote et al, 2005).

The profile of methylation also differed in various localizations of head-neck tumours. Hypermethylation of *RASSF1* occurred more frequently in cutis of head-neck when compared to other localizations. In contrast, *p16* and *p14* hypermethylation was not detected in cutis, but was frequent in carcinomas of larynx and hypopharynx. It was shown previously, that *p16* and *p14* hypermethylation correlates with tumour size, node involvement, high stage and grade (Ishida et al, 2005, Weber et al, 2003, Steinmann et al, 2009). Also, hypermethylation in ≥1 gene promoters is more frequent in higher

clinical stages, and high grade tumours (Steinmann et al, 2009). In our study on head-neck cancer, methylation of *CDKN2* locus (genes *p16* and *p14*) was observed in three late clinical stage tumours. Also, hypermethylation in ≥1 gene promoters was more common in late stage head-neck carcinomas.

Salivary gland carcinomas do not show direct association to tobacco smoke exposure. Though, belonging to a group of head-neck tumours, salivary gland carcinomas might share similar genetic and epigenetic alterations. Promoter hypermethylation of *MGMT* is frequent in head-neck carcinomas. In various neoplasms, transcriptional silencing of the gene is postulated to be the predominant mechanism for the loss of MGMT protein expression (Esteller et al, 2004). There is data that the methylation status of a single CpG site in promoter region may differ, which, in turn, may have different consequences for transcriptional silencing of the gene (Illingworth et al, 2009). In our study, highly sensitive pyrosequencing method was used to evaluate methylation level in *MGMT* promoter in salivary gland carcinomas and histologically normal tissues. We determined methylation status in tumour DNA (27.8%) to be elevated when compared to histologically normal tissues. Furthermore, we found the significant correlation of *MGMT* promoter methylation and the loss of protein expression in tumours. To date, this association is shown for stomach, breast, prostate, colon, brain and head-neck cancers (Bae et al, 2002, Esteller et al, 2000, Sharma et al, 2010, Zuo et al, 2004). In previous study, the relationship between aberrant *MGMT* promoter methylation and protein loss in salivary gland carcinomas was not recognized (Williams et al, 2006). Also, *MGMT* promoter hypermethylation was not detected in a group of 78 salivary gland carcinomas using a quantitative MSP method (Durr et al, 2010). The discrepancy to our study could be explained by the different CpG islands within *MGMT* promoter investigated.

Recent observations highlight the importance of *MGMT* as a biomarker for the responsiveness of treatment with alkylating chemotherapy in glioma, lymphoma patients. Patients having tumours with *MGMT* promoter hypermethylation and subsequent MGMT protein loss have better survival chances. *Vice versa*, unmethylated *MGMT* promoter and the expression of MGMT protein results in the resistance of tumours to alkylating agents. As to date, non-alkylating chemotherapy is administered to salivary gland carcinoma patients, the survival dependence on the status of *MGMT* promoter methylation and MGMT protein expression could not be examined in this study. However, the observed associations between the loss of MGMT protein expression and worse clinical parameters suggest that *MGMT* promoter hypermethylation and MGMT protein loss is an informative prognostic marker of salivary gland cancer.

CONCLUSIONS

1. In lung tumours (n=212), the frequencies of promoter hypermethylation of the *p16*, *RARB*, *RASSF1*, *MGMT*, *DAPK1* genes are 15 - 31%; the most informative epigenetic markers are hypermethylation of *p16*, *RARB* and *RASSF1*. The prevalence of *p16* hypermethylation is significantly higher in males than females (p=0.018); squamous cell carcinomas than adenocarcinomas (p=0.025); Aberrant methylation is associated with *TP53* gene mutations (p=0.027). Promoter methylation of *p16* reflects the effect of tobacco smoke and is observed in lung tumours from smokers or neversmokers with a systemic second-hand tobacco exposure, only.
2. In head-neck tumour scrapings (n=31), the frequencies of promoter hypermethylation of the *p16*, *p14*, *RARB*, *RASSF1*, *MGMT* genes are 19 - 50%; the most informative epigenetic marker is hypermethylation of *MGMT*. Promoter hypermethylation of *p16* is observed in scrapings of oral mucosa from smokers, only, and the prevalence of hypermethylation in ≥1 gene promoter is higher in specimens obtained from smokers than neversmokers (p=0.047).
3. The frequency of *MGMT* promoter methylation in salivary gland carcinomas (n=36) is higher than in histologically normal tissues (n=19) (28% and 16%, respectively). In tumours, epigenetic changes in gene *MGMT* are associated with loss of MGMT protein expression (p=0.021). The loss of MGMT protein expression in tumours is characteristic for patients with poor prognosis (high grade, certain histologic types, lymph node involved tumours).

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1. "Naviką slopinančių genų promotoriaus hipermetilinimas galvos ir kaklo navikų ląstelėse", Lithuanian biochemists' society conference, Lithuania 2008.
2. "Significance of tumor suppressor gene hypermethylation in head-neck tumours", 2-nd annual COST BM0703 conference, Sweden 2009.
3. "Significance of tumor suppressor gene hypermethylation in head-neck tumours", conference "Molecular pathology for diagnosis and treatment", Lithuania 2010.

Participation in conferences

1. Sonata Jarmalaite, Asta Scesnaite, Fredrik Nyberg, Simone Benhamou, Paolo Boffetta, Kirsti Husgafvel-Pursiainen. "Aberrant promoter methylation in lung cancer from never-smokers with and without tobacco smoke exposure", 5-th annual AARC conference, USA 2006.
2. Asta Scesnaite, Sonata Jarmalaite, Reetta Holmila, Tuula Suitiala, Kirsti Husgafvel-Pursiainen. "Smoking induced gene hypermethylation in non-small cell lung cancer", 38-th annual European environmental mutagen society conference, Croatia 2008.

3. Asta Scesnaite, Sonata Jarmalaite, Stefan Schwarz, Regine Schneider-Stock. “Prognostic value of tumor suppressor gene inactivation in salivary gland carcinomas”, COST BM0703 conference, UK 2010.
4. Asta Scesnaite, Sonata Jarmalaite, Fredrik Nyberg, Simone Benhamou, Paolo Boffetta, and Kirsti Husgafvel-Pursiainen. "Smoking induced gene promoter hypermethylation in non-small cell lung cancer", COST BM0703 conference, Italy 2010.

Supplementary table 1. The frequencies of promoter methylation in lung tumours.

Parameters	**Total, n (%)**	***p16*, n (%)**	***RARB*, n (%)**	***RASSF1*, n (%)**	***MGMT*, n (%)**	***DAPK1*, n (%)**
Sex						
Female	83	8 (9.6)	33 (39.8)	20 (24.4)[1]	13 (15.7)	22 (26.5)
Male	129	36 (27.9)	33 (25.6)	41 (31.8)	19 (14.7)	20 (15.5)
p value		**0.002**	**0.044**	0318	1.000	0.076
Histology						
Adenocarcinoma	103	13 (12.6)	40 (38.8)	27 (26.5)[1]	12 (11.7)	22 (21.4)
Squamous cell carcinoma	79	27 (34.2)	22 (27.9)	22 (27.9)	15 (18.9)	15 (18.9)
Other	30	4 (13.3)	4 (13.3)	12 (40.0)	5 (16.7)	5 (16.7)
p value: [2]		**0.001**	0.163	0.967	0.243	0.839
[3]		1.000	**0.012**	0.232	0.654	0.782
Smoking status						
Neversmoker	47	5 (10.6)	20 (42.6)	11 (23.9)[1]	5 (10.6)	11 (23.4)
Smoker	165	39 (23.6)	46 (27.9)	50 (30.3)	27 (16.4)	31 (18.8)
p value		0.073	0.086	0.514	0.471	0.609
***TP53* gene**						
Wild-type	133	21 (15.8)	42 (31.6)	33 (25.0)[1]	15 (11.3)	26 (19.6)
Mutant	79	23 (29.1)	24 (30.4)	28 (35.4)	17 (21.5)	16 (20.3)
p value		**0.034**	0.980	0.145	0.072	1.000
Total, n (%)	212	44 (20.8)	66 (31.1)	61 (28.9)	32 (15.1)	42 (19.8)

[1] - data for 1 case not available, [2] - squamous cell carcinoma *versus* adenocarcinoma, [3] - other localizations *versus* adenocarcinoma.

Supplementary table 2. The frequencies of promoter methylation in head-neck cancer cases and controls.

Parameters	**Total, n**	***p16*, %**	***p14*, %**	***RARB*, %**	***RASSF1*, %**	***MGMT*, %**	**ORH, %**
Age, years							
<60	22	18	19	25	12	44	59
≥60	20	20	25	11	17	50	70
Sex							
Female	10	0	20	13	14	50	40
Male	32	25	23	20	14	44	72
Head-neck cancer							
No	11	9	27	0	0	25	45
Yes	31	23	20	20	19	50	71
Localization							
Cutis of head-neck region	5	0	0	40	60	33	60
Larynx, hypopharynx	14	36	39	8	10	56	79
Other	12	17	8	33	8*[1]	50	67
Smoking status							
Neversmoker	9	11	11	22	14	50	33
Smoker	30	23	21	18	15	47	73*
Stage							
I-II	12	8	0	27	27	33	50
III-IV	19	32	32	21	13	58	84*[2]
HPV							
No	29	21	21	20	8	50	62
Yes	13	15	23	15	27	38	69

ORH - overall rate of hypermethylation, * - statistically significant differences, *[1] - significant when compared to cutis of head-neck, *[2] - borderline significance (p=0.056).

Supplementary table 3. The association between MGMT protein loss and various clinical, pathological parameters in salivary gland carcinoma patients.

Parameters	**Total n**	**Loss of MGMT protein expression, n (%)**	**p value**
Age, years	286		
MGMT lost	112	64.5	**0.007**
MGMT expressed	174	57.6	
Sex	286		**0.046**
Female		50/150 (33.3)	
Male		61/136 (44.9)	
Localization	286		NS
Parotid glands		82/200 (41)	
Submandibular glands		13/42 (31)	
Minor salivary glands		16/43 (37.2)	
Sublingual glands		0/1 (0)	
Histology	268		**0.002**
Acinic cell carcinoma		8/41 (19.5)	
Adenoid cystic carcinoma		11/46 (23.9)	
Mucoepidermoid carcinoma		15/42 (35.7)	
Squamous cell carcinoma, adenocarcinoma, not otherwise specified, salivary duct carcinoma		52/101 (51.5)	
Myoepithelial carcinoma, malignant mixed tumour		10/21 (47.6)	
Basal cell adenocarcinoma, epithelial-myoepithelial carcinoma, polymorphous low-grade adenocarcinoma		6/17 (35.3)	
Stage	283		NS
I		19/66 (28.8)	
II		22/62 (35.5)	
III		26/62 (41.9)	
IV		44/93 (47.3)	
Grade	285		**<0.001**
I		21/88 (18.9)	
II		25/68 (22.5)	
III		65/129 (58.6)	
pT	238		NS
1		24/76 (31.6)	
2		41/94 (43.6)	
3		27/68 (39.7)	
Metastases	281		NS
No		104/258 (40.3)	
Yes		6/23 (26.1)	

Node involvement	270		**<0.001**
No		61/190 (32.1)	
Yes		46/80 (57.5)	
Death	286		**0.012**
No		59/178 (33.1)	
Yes		52/108 (48.1)	

NS - statistically not significant.

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SANTRAUKA

Plaučių bei galvos-kaklo navikai - vienos labiausiai paplitusių ir didžiausiu mirtingumu pasižyminčių onkologinių ligų Lietuvoje ir pasaulyje. Svarbiausias šių susirgimų etiologinis veiksnys yra rūkymas. Tabako sudėtyje esantys kancerogenai ir prokancerogenai sukelia genetines ir epigenetines pažaidas. Šiame darbe siekėme įvertinti epigenetinių (DNR metilinimo) pažaidų dažnį ir spektrą su rūkymu susijusiuose plaučių ir galvos-kaklo navikuose.

Metilinimui jautria PGR ir pirosekvenavimo metodais tyrėme DNR metilinimo pažaidas naviką slopinančiuose genuose *p16*, *p14*, *RARB*, *RASSF1*, *MGMT* ir *DAPK* plaučių (n=212), galvos-kaklo (n=31), seilių liaukų (n=36) karcinomose, neonkologinių ligonių burnos epitelio nuograndose (n=11) ir histologiškai nepakitusiuose seilių liaukų audiniuose (n=19). Imunohistochemijos metodu tyrėme MGMT baltymo raiškos pakitimus seilių liaukų mėginiuose (n=287). Plaučių navikuose nustatėme šiuos genų promotorių hipermetilinimo dažnius: *p16* - 20,8%, *RARB* - 31,1%, *RASSF1* - 28,9%, *MGMT* - 15,1% ir *DAPK1* - 19,8%. Geno *p16* promotoriaus hipermetilinimo tikimybė yra didesnė vyrų nei moterų plaučių karcinomose (p=0,018), plokščialąstelinėse plaučių karcinomose nei adenokarcinomose (p=0,025). DNR metilinimo pažaidos ≥1 geno promotoriuje yra susijusios su mutacijomis *TP53* gene (p=0,027). Nerūkančių, bet veiktų aplinkos tabako dūmais, plaučių vėžio ligonių navikuose metilinimo indeksas ir geno *p16* epigenetinių pažaidų dažnis yra artimas metilinimo indeksui ir pažaidų dažniui, nustatomam rūkančiųjų navikuose. Galvos-kaklo karcinomų nuograndų DNR nustatėme šiuos genų promotorių hipermetilinimo dažnius: *p16* - 23%, *p14* - 20%, *RARB* - 20%, *RASSF1* - 19%, *MGMT* - 50%. ≥1 geno promotoriaus hipermetilinimo tikimybė yra didesnė rūkančių negu nerūkančių asmenų mėginiuose (p=0,047). Seilių liaukų karcinomose ir histologiškai nepakitusiuose seilių liaukų audiniuose promotoriaus hipermetilinimą nustatėme 27,8% ir 15,8% atvejų, atitinkamai. Epigenetinės pažaidos geno *MGMT* promotoriuje seilių liaukų navikuose koreliuoja su MGMT baltymo raiškos praradimu (p=0,021). MGMT baltymo raiškos praradimas yra būdingas blogos prognozės (blogos diferenciacijos, išplitusiems į sritinius limfmazgius, tam tikrų histologinių tipų) seilių liaukų navikams.

Mūsų tyrimas prisideda prie gilesnio supratimo apie tabako kancerogenų sukeltus molekulinius pokyčius plaučių ir galvos-kaklo navikuose. Metilinimo pažaidos atspindi rūkymo ar poveikio aplinkos tabako dūmais žalą. Genų promotoriaus sekų hipermetilinimo analizė DNR, išskirtoje iš didelės rizikos (rūkančiųjų) asmenų burnos ertmės nuograndų galėtų būti tinkama ankstyvos galvos-kaklo navikų rizikos vertinimo priemonė. Seilių liaukų navikuose geno *MGMT* promotoriaus hipermetilinimas lemia MGMT baltymo raiškos praradimą. Šis pokytis yra reikšmingas seilių liaukų navikų patogenezei.

Darbo tikslas

Pagrindinis darbo tikslas - nustatyti plaučių bei galvos-kaklo navikams būdingus DNR metilinimo pakitimus; įvertinti šių epigenetinių žymenų sąsajas su klinikiniais ir demografiniais rodikliais, susieti su ligonio rūkymo įpročiais bei sisteminiu kontaktu su aplikos tabako dūmais.

Darbo uždaviniai

1. Plaučių navikuose ištirti genų *p16, RARB, RASSF1, MGMT, DAPK1* promotoriaus DNR metilinimo pakitimus ir įvertinti sąsajas tarp epigenetinių pažaidų dažnio bei demografinių, klinikinių, rūkymo (aktyvaus ar pasyvaus) intensyvumo rodiklių.
2. Galvos-kaklo srities navikų ir onkologine liga nesergančių asmenų burnos epitelio nuograndose ištirti DNR metilinimo pakitimus genuose *p16, p14, RARB, RASSF1, MGMT* ir įvertinti sąsajas tarp epigenetinių pažaidų dažnio bei demografinių, klinikinių, rūkymo intensyvumo rodiklių.
3. Seilių liaukų navikuose ir histologiškai nepakitusiuose seilių liaukų audiniuose ištirti geno *MGMT* promotoriaus DNR hipermetilinimo dažnį ir baltymo MGMT raiškos pakitimus. Įvertinti šių pakitimų sąsajas su demografiniais ir klinikiniais rodikliais bei susieti geno promotoriaus metilinimą su baltymo raiška.

Ginamieji teiginiai

1. DNR metilinimo pažaidos plaučių ir galvos-kaklo navikuose yra ligos ir tabako dūmų poveikio (tiesioginio ir netiesioginio) žymenys.
2. Galvos-kaklo vėžiui būdingi DNR metilinimo žymenys gali būti tiriami ligos pažeisto burnos epitelio nuograndose ir tinka ankstyvam vėžio rizikos nustatymui.
3. Seilių liaukų navikuose geno *MGMT* promotoriaus hipermetilinimas lemia MGMT baltymo raiškos praradimą; šis pokytis reikšmingai įtakoja ligos eigą.

Darbo mokslinis naujumas

Šiame darbe nagrinėjama nauja, sparčiai besiplėtojanti mokslo sritis - vėžio epigenetika. Analizuojamas molekulinių pakitimų spektras nesmulkialąstelinėse nerūkančiųjų ir rūkančiųjų asmenų plaučių karcinomose, galvos-kaklo srities navikuose. Panašių tyrimų, analizuojančių vėžio genų promotorių metilinimo būklę nerūkančiųjų plaučių karcinomose, pasaulyje nedaug (Kim et al, 2001, Jarmalaite et al, 2003) dėl nedidelio nerūkančių ir sergančių plaučių vėžiu ligonių skaičiaus. Žymiai daugiau tyrimų atlikta rūkančiųjų ir metusių rūkyti plaučių karcinomose (Kim et al, 2001, Belinsky, 2004). Darbe įvertinome genų promotoriaus hipermetilinimo sąsajas su tiesioginiu ar netiesioginiu tabako dūmų poveikiu. Vieni iš nedaugelio parodėme, kad metilinimo pažaidos *p16* geno promotoriuje yra būdingos tik rūkančiųjų arba ATD veiktų plaučių vėžio ligonių navikams.

Molekulinių su patogeneze susijusių pakitimų tyrimų galvos-kaklo karcinomose gausu, tačiau dėl skirtingos anatominės navikų lokalizacijos, tyrimų rezultatai įvairūs. Dažnai pateikiami prieštaringi, sunkiai apibendrinami duomenys. Mūsų darbe pavyko atrinkti informatyviausią galvos-kaklo navikų žymenį - *MGMT* geno promotoriaus hipermetilinimą. Taip pat parodėme, kad DNR metilinimo pažaidos atspindi tabako kancerogenų daromą žalą.

Šiame darbe pirmą kartą pateikiami retų seilių liaukų navikų tyrimai pakankamai didelėje mėginių imtyje. Tiriamas *MGMT* geno promotoriaus metilinimas ir analizuojama MGMT baltymo raiška homogeniškoje galvos-kaklo navikų kolekcijoje. Literatūroje yra duomenų apie baltymus - seilių liaukų navikų žymenis, tačiau epigenetiniai pakitimai navikuose, o ypač histologiškai nepakitusiuose audiniuose tiriami retai. Dėl nedidelio seilių liaukų vėžio dažnio mūsų krašte molekulinių žymenų analizės

Lietuvoje iki šiol atlikta nebuvo. Darbe parodėme, kad MGMT baltymo raiška seilių liaukų navikuose koreliuoja su blogai prognozei būdingais klinikiniais rodikliais.

Šiuo darbu siekėme papildyti fundamentalias mokslo žinias apie DNR metilinimo ypatumus plaučių ir galvos-kaklo navikuose. Tyrimai taip pat yra vertingi praktikoje, kadangi DNR metilinimo žymenys diegiami į molekulinės vėžio diagnostikos sistemas.

Darbo mokslinė ir praktinė reikšmė

Genetinės ir epigenetinės pažaidos naviką slopinančiuose genuose (NSG) yra tiesiogiai susijusios kancerogeniniu aplinkos poveikiu ir įtakoja ligos genezę. Šių pažaidų analizė suteikia informacijos apie onkologinės ligos vystymosi riziką, eigą ar galimą jautrumą gydymui. Naujų biožymenų identifikavimas padeda kurti naujos kartos gydymo strategijas, nukreiptas į konkrečius molekulinius taikinius.

Darbe, naudojant senesnius patikimus (MSP) ir naujus (pirosekvenavimas) molekulinės biologijos tyrimo metodus, plaučių ir galvos-kaklo navikų mėginiuose analizavome DNR metilinimo pažaidas naviką slopinančiuose genuose *p16*, *p14*, *RARB*, *RASSF1*, *MGMT* ir *DAPK1*. DNR metilinimo pažaidų tyrimu siekėme prisidėti prie molekulinių žymenų sistemos, naujos kartos prevencijos ir gydymo strategijos kūrimo, pasitarnausiančios ankstyvai diagnostikai, prognozės vertinimui, tinkamiausio gydymo plano parinkimui. Plaučių ir seilių liaukų navikų tyrimuose nustatėme epigenetinių pažaidų ir baltymo raiškos sąsajas su klinikiniais, demografiniais rodikliais. Parodėme, kad DNR metilinimo pažaidų analizė gali padėti įvertinti vėžio riziką, o baltymo raiškos tyrimas gali pagelbėti prognozuoti ligos eigą.

Epigenetinių pažaidų analizė pasaulyje jau pradėta diegti į įprastines diagnostines sistemas, ypač kuriant neinvazyvius ar mažai invazyvius įvairių lokalizacijų navikų diagnostikos būdus, didelės rizikos asmenų prevencinės patikros programas. Galvos-kaklo mėginių tyrimu parodėme, kad metilinimo pažaidos NSG promotoriuose galima aptikti navikų ir burnos epitelio nuograndose (neinvaziniais metodais paimtoje biologinėje medžiagoje), tyrimas yra informatyvus, o metodo jautrumas pakankamas. Tai galėtų būti papildoma vertinimo priemonė įprastiems diagnostikos metodams.

Šiame darbe atlikti tyrimai praplečia žinias apie epigenetinių pažaidų svarbą plaučių, galvos-kaklo, seilių liaukų vėžio patogenezei, suteikia naujos informacijos apie nerūkančiųjų plaučių vėžio formavimosi (epi)genetinius ypatumus. Remiantis šiais darbais buvo pradėta epigenetinių žymenų paieška Lietuvių krūties ir prostatos vėžio ligonių biologinėje medžiagoje, o užsienio mokslo centruose įgyta patirtis perkelta diegiant metodikas į laboratoriją Lietuvoje.

Išvados

1. Plaučių navikuose (n=212) genų *p16*, *RARB*, *RASSF1*, *MGMT*, *DAPK1* promotoriaus DNR hipermetilinimo dažniai yra nuo 15 iki 31%; informatyviausi epigenetiniai žymenys yra genų *p16*, *RARB* ir *RASSF1* hipermetilinimas. Geno *p16* promotoriaus hipermetilinimo tikimybė yra didesnė vyrų nei moterų plaučių karcinomose (p=0,018); plokščialąstelinėse plaučių karcinomose nei adenokarcinomose (p=0,025); DNR metilinimo pažaidos siejasi su mutacijomis *TP53* gene (p=0,027). Geno *p16* hipermetilinimas atspindi tabako dūmų poveikį ir aptinkamas tik rūkančiųjų ar sistemingai aplinkos tabako dūmais veiktų asmenų plaučių navikuose.
2. Galvos-kaklo navikų (n=31) nuograndose genų *p16, p14, RARB, RASSF1, MGMT* promotoriaus DNR hipermetilinimo dažniai yra nuo 19 iki 50%; informatyviausias

epigenetinis žymuo - geno *MGMT* hipermetilinimas. Geno *p16* hipermetilinimas aptinkamas tik rūkančiųjų burnos epitelio nuograndose, o ≥1 geno hipermetilinimo tikimybė yra didesnė rūkančiųjų negu nerūkančių asmenų mėginiuose (p=0,047*).*

3. Seilių liaukų navikuose (n=36) geno *MGMT* hipermetilinimo dažnis didesnis negu histologiškai nepakitusiuose seilių liaukų audiniuose (n=19) ir atitinkamai yra 28% ir 16%. Navikuose epigenetinės pažaidos geno *MGMT* promotoriuje koreliuoja su MGMT baltymo raiškos praradimu (p=0,021); šis pokytis yra būdingas blogos prognozės (blogos diferenciacijos, išplitusiems į sritinius limfmazgius, tam tikrų histologinių tipų) seilių liaukų navikams.

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Internships

Finnish Institute of Occupational Health, Finland, 2006, 2008.
University Erlangen-Nurnberg, Germany, 2009, 2010.

Workplace

2007-2011 research assistant in Department of Botany and Genetics, Faculty of Natural sciences, Vilnius university.

Scientific interests

Cancer biology and genetics, epigenetics, molecular biomarkers.

CURRICULUM VITAE

Asmeninė informacija

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Išsilavinimas

2000-2004 Biologijos bakalauro laipsnis, Vilniaus universitetas, Gamtos mokslų fakultetas.
2004-2006 Biologijos magistro laipsnis, Vilniaus universitetas, Gamtos mokslų fakultetas.
2006-2010 Biomedicinos mokslų srities, Biologijos mokslų krypties doktorantė, Vilniaus universitetas, Gamtos mokslų fakultetas.

Stažuotės

Suomijos profesinės sveikatos institutas, Suomija, 2006 m., 2008 m.
Erlangen-Nurnberg universitetas, Vokietija, 2009 m., 2010m.

Darbovietė

2007-2011 Vilniaus universitetas, Gamtos mokslų fakultetas, Botanikos ir genetikos katedra, jaunesnioji mokslo darbuotoja.

Moksliniai interesai

Vėžio biologija ir genetika, epigenetika, molekuliniai žymenys.