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The value of *SHOX2* methylation test in peripheral blood samples used for the differential diagnosis of lung cancer and other lung disorders

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Methylation of the cytosine residues within the CpG dinucleotides plays an important role in the fundamental cellular processes, human diseases and even cancer. The DNA methylation represents a very stable sign and therefore may be used as a valuable marker for cancer screening. Epigenetic cancer biomarkers are independent of classical morphology and thus show extensive potential to overcome the limitations of cytology. Several epigenetic cancer markers have been reported to be detectable in body fluids such as bronchial aspirate, sputum, plasma and serum.

Short stature homeobox gene 2 (*SHOX2*) encodes a homeo-domain transcription factor, which has been identified as a close homologue of the *SHOX* gene and both genes are involved in skeletogenesis and heart development. Methylation of *SHOX2* gene has been shown to be present at high prevalence in carcinomas of lung, however may also be used to identify other tumour entities.

In the presented study, we have compared suitability of two types of material associated with lung cancer for the detection of *SHOX2* methylation. We have confirmed that methylation of *SHOX2* gene represents reliable marker of lung malignancies. The parallel tests in the blood plasma revealed that it may represent a good alternative material for testing of the *SHOX2* methylation, making the test available to patients who are unable to undergo bronchoscopy.

Key words: DNA methylation test, SHOX2, lung cancer, bronchial lavage, blood plasma

Lung cancer represents the most common malignancy and the main cause of cancer-related death worldwide [1]. Onset of the lung cancer is the result of many factors, genetic, epigenetic and environmental ones. Majority of lung cancers, approximately 80-90%, could be attributed to cigarette smoking [2]. Nowadays, there is sufficient evidence to infer a causal relationship between smoking and lung cancer. Smoking causes genetic changes in cells of the lung that ultimately lead to the development of lung cancer. In consequence, 85% of all lung cancer deaths are estimated to be attributed to cigarette smoking [3]. Nevertheless, about 10-20% of lung cancer cases occur in non-smokers [4], what clearly points to the fact that lung cancer risk is not explainable simply by cigarette smoking. Thus, besides age and gender, other risk factors, such as environmental exposure, lung function, or genetic markers need to be taken into account [3]. In 2005, the mortality of lung cancer in Slovak men was estimated at 50.3 (out of 100 000) and in Slovak women 7.6 (out of 100 000) [5].

Based on histology, lung cancers can be divided in two main histological groups, non-small cell (85%) and small cell lung cancer. This grouping has major impact on further clinical management of the patients, treatment and also on the prognosis of the disease [6]. One of the most important factors contributing to the successful and effective treatment is the early detection of the disease, which may have major impact on the outcome. However, imaging or cytology strategies often fail to detect early stages of lung cancer [7]. Lack of effective tools to diagnose lung cancer at an early stage (that means before it has spread to regional lymph pulmonary nodule or metastasized beyond the lung) results in a 5-year mortality rate from 80% to 85% [8]. Current efforts are focused on identification, selection and validation of new non-invasive or

minimally invasive markers which might assist early diagnosis, prognosis of the defined disease and prediction of response to treatment.

Recent data support the view that genetic and epigenetic factors play an important causal role in carcinogenesis [9]. Most common events observed in the lung cancer are epigenetic alterations, such as promoter DNA methylation, which is often associated with gene silencing.

Epigenetic abnormalities are presented in all human cancers. Cancer cells globally present DNA hypomethylation or also hypermethylation of tumor suppressor genes [10]. Well-studied gene examples are hypermethylation of *p16* [11], H-cadherin, *RASSF1A*, *APC* and *DAPK1* genes [12]. Compared to other molecular markers, such as mRNA or proteins, DNA methylation represents a chemically stable marker. It is a covalent DNA modification which can be easily detected by standard molecular biology methods and which thus might constitute such an early indicator of neoplastic transformation. This assumption is based on the fact of high (above average) level of p16 methylation observed in pulmonary hyperplasia (17%), dysplasia (24%) and lung carcinoma *in situ* (50%) [13].

Information about abnormal DNA methylation can be useful in disease phenotyping, more precise histological typing, determining the tumor aggressiveness, prediction of therapy efficiency and even detection of risk of cancer development. Importantly, it can be detected in many different types of tissues and samples, including tumor tissues, cancer cells in body fluids, cell-free tumor DNA in blood plasma. Lung cancer-associated aberrant DNA methylation is often detected in patient´s sputum [14], bronchoalveolar lavage [15] or saliva [16].

SHOX2 gene (short stature homeobox 2 gene) is localized on the 3rd chromosome (3q25.32), is 10 kb in size and comprises of 7 exons encoding a 319 amino-acid protein. It is a member of the homeobox genes family that encodes proteins with specific 60-amino acid DNA binding domain. Two splicing variants of *SHOX2* transcripts are known: A (993 bp) and B (570 bp) [17]. Two huge CpG islands are located within the *SHOX2* gene, one covering 1 kb at the 5´ end, another 500-bp island is located at the 3´ end. The homeobox genes have been characterized as transcription factors involved in pattern formation in invertebrates as well as vertebrates. *SHOX2* has been shown to play a major role during skeletogenesis and heart development [18]. The *SHOX2* gene is exclusively expressed in the anterior mesenchyme region of the developing secondary palate, with the highest expression during the early stage of palate development [19]. Generally, the homeobox genes represent a pseudoautosomal locus that is thought to be responsible for idiopathic short stature, and for short stature phenotype of Turner syndrome patients.

The *SHOX2* genes, together with *SEPT9*, are the methylation markers used in routine diagnostics of lung or colorectal cancer respectively. Both markers have been validated on large numbers of patients and have been reported to have high specificity and sensitivity. Aberrant DNA methylation of *SHOX2* is a hallmark of lung tumors and testing the methylation has been validated in bronchial lavage samples from patients with suspected lung cancer, including those with negative cytopathological result and those with no visual detection of the tumor [20].

Kneip et al. [21] have reported that lung cancer-associated DNA methylation of *SHOX2* gene can also be successfully detected in blood plasma with sensitivity of 60% and specificity of 90%. Begum et al. [22] tested the methylation status of 6 different genes (*APC, CDH1, MGMT, DCC, RASSF1A, AIM1*). Their results suggest 100% specificity of *DCC* gene methylation and they were able to correctly identify all lung cancer patients using this method. However, these results need to be confirmed in longitudinal studies.

Since *SHOX2* methylation in blood plasma was previously reported as sensitive and specific diagnostic test, it is reasonable to consider and explore in more detail the benefits and limitations of this noninvasive alternative to the current bronchial lavage test [21]. Actually, CE marked IVD molecular test based on the *SHOX2* DNA methylation in lavage samples is commercially available in Europe [20]. On the other hand it is still not clear if the blood plasma *SHOX2* methylation test may completely substitute the lavage testing in the diagnostics and even if the presence of *SHOX2* methylation in blood plasma sufficiently reflects the presence of tumor. The focus of the study was to evaluate evidence for using of blood plasma methylation method in diagnosing patients with lung cancer, either as a replacement for or an addition to currently used bronchial lavage.

The main objective of our study was to determine the diagnostic performance of *SHOX2* methylation tests in parallel samples of bronchial lavage and peripheral blood from the same patient. The secondary objective was to evaluate the degree of agreement between these two methods.

Materials and methods

Samples. We can define our study as a type of observational case-control study with data collected in a cross-sectional manner. The cases (positive group) and controls (negative group) were selected according to the presence or absence of malignant disease defined by cytology or histology analysis.

In our study, we recruited patients undergoing bronchoscopy at the Department of Pneumology and Phtiseology of University Hospital in Bratislava for suspicion of lung cancer or alternate non-cancer disease. From all eligible patients parallel samples of bronchial lavage and peripheral blood were obtained. Bronchial lavages were collected into Saccomano fixative in the ratio 1:1, specifically 1 ml of lavage was mixed with 1 ml of fixative, and stored at 4°C until further use. Peripheral blood was collected in the volume of 7 ml into EDTA tubes and centrifuged. Plasma was collected and stored at -20°C until analysis (no more than 2 weeks). Totally, 69 parallel samples were collected at Department of Clinical Genetics, St. Elizabeth

Cancer Institute between January 2014 and December 2014, with addition time for data collection in January and February of 2015. Based on the cytological or histological analysis, 38 samples were positive for malignant lung disease, the rest of the samples (n=31) were considered negative for malignancy. These patients underwent bronchoscopy and lavage due to other non-malignant lung condition and no malignancy was found in their samples.

DNA isolation. DNA was isolated using commercial Epi proLung BL DNA preparation kit (Epigenomics) for bronchial lavages and Epi proColon Plasma Quick kit (Epigenomics) for peripheral blood samples according to manufacturer´s recommendations. Bisulfide conversion of purified DNA was performed by addition of bisulfite reagent and denaturation buffer, at 85°C for 45 minutes in thermo-shaker at 1000 rpm. Treated DNA was stored at 4°C and analyzed within 24 hours.

Real time PCR analysis. The real-time PCR analysis was performed using the Epi proLung BL real-time PCR kit (Epigenomics) on the ABI 7500 Fast real-time PCR instrument (Life Technologies) using specific temperature profile (95°C/15 min., 40 cycles of 56°C/30 sec., 95°C/15 sec.). Sequences of the probes and primers were previously published [23]. The PCR analysis uses two target-specific probes; one for methylation-prone sequence of *SHOX2* gene labeled with FAM and second targeting methylation-independent sequence in *ACTB* gene labeled with JOE. The PCR was performed in two parallel reactions for each sample. Calibrator and positive/negative control samples were analyzed in each run as well (Control Work-flow kit, Epigenomics). Threshold and baseline settings were set to 0.01, baseline start at 3 and baseline stop at 15 for *ACTB* and 20 for *SHOX2*. The run was considered valid if the cycle threshold (C_T) value for *ACTB* in positive sample was below 31 and C_T for *SHOX2* was below 37; the C_T for *ACTB* in negative control was between 28-37 and C_T for *SHOX2* above 37. Lavage samples were considered positive if C_T for *ACTB* was below 29 and blood samples were considered positive if C_T for *ACTB* was below 35. Calibrator sample was considered positive if C_T for *ACTB* was below 32. Methylation data analysis was performed using $\Delta\Delta C_T$ method according to the Epi proLung BL kit manual. In the first step ΔC_T for each sample as well as calibrator was calculated using the following equation: C_T(*SHOX2*) – C_T(*ACTB*). The ΔΔCt value was calculated by subtracting ΔC_T of each sample from ΔC_T of calibrator. If the $\Delta\Delta C_T$ value was lower than 9.5, samples were considered positive for methylated *SHOX2*.

Statistical analysis. The diagnosis of lung cancer (selection of cases) was based on biopsy/cytology results. Descriptive and bivariate statistics were performed on all patients' characteristics. Both groups were generally balanced with regard to gender ($P > 0.05$) and unbalanced with regard to the age distribution ($P < 0.05$). Agreement analysis was used to evaluate the degree of agreement and disagreement of both methods over categories in 2x2 tables. Diagnostic performance of each single method was evaluated using the biopsy/cytology results. Cohen's Kappa, Maxwell's chi-square and McNemar's statistics were used to test for agreement, disagreement and significant differences. In broad terms a Kappa below 0.2 indicates poor agreement and a Kappa above 0.8 indicates very good agreement beyond chance. Maxwell's chi-square statistic tests for overall disagreement between the two methods' readings. The general McNemar statistic tests for asymmetry in the distribution of subjects about which the readings disagree, i.e. disagreement more over some categories of response than others.

Statistical modeling. We used multivariable logistic regression for construction and evaluation of lung cancer risk models. The available clinical variables included age, and the following dichotomous variables: gender (1 male, 0 female), smoking status (1 smoker/former smoker, 0 non-smoker), cell type (1 SCLC, 0 otherwise, i.e. NSCLC all subtypes). The model analysis and selection was made using goodness of fit tests (Pearson, deviance and Hosmer-Lemeshow tests) and Akaike's information criterion. Because of different age distribution between cases and controls all tested models were adjusted to age. Effect size for significant or clinically important explanatory variables was estimated using odds ratio (OR) and a corresponding 95% confidence interval (95% CI). Receiver Operating Characteristic (ROC) curve analysis was used to define detection cut-off points for analyzed diagnostic tests represented by the multivariable models. In a ROC curve sensitivity (probability of positive test when disease is present) is plotted against 1-specificity (probability of negative test when disease is absent). Resulting ROC curves were compared using AUC criterion (the area under the curve). The accuracy, sensitivity, specificity, positive predictive value (PPV, i.e. post-test likelihood of disease), and negative predictive value (NPV, i.e. post-test likelihood of of no disease), all along with their 95% CI, were also calculated for the bronchial lavage and peripheral blood tests, as well as for the models. For each of the models, patients that had a probability of lung cancer ≥ 0.5 were classified as having lung cancer, and patients with a probability < 0.5 were classified as not having lung cancer. The final model was composed of four explanatory variables that most contributed to the risk.

All tests were conducted at significance level of 5% using StatsDirect 2.8.0 software.

Results

Overall methylation analysis. Samples were collected from 69 patients, specifically 49 men and 20 women. Average age of the patients was 60.4 years of age; 54.1 years for patients with diagnoses other than lung cancer (further referred as negative) and 66.7 years for patients with confirmed lung cancer. There were 16 non-smokers (23.2%), 3 in positive group and 13 in negative group. The rest of the patients (76.8%) were active or former smokers (n=53), 35 with detected malignancy and 18 without lung cancer (Table 1). Two types of material were

Table 1. Distribution of the tested group of samples according to selected categories and based on tumor presence.

Category	Samples	Tumor samples					P-value	Tumor samples total	Non-tumor samples total	P-value
		Stage I	Stage II/ LimDis	stage III	stage IV/ ExDis	Undetermined staging	Fisher exact test			Fisher exact test
Total		1	4	8	22	3	n.a.	38	31	n.a.
Type of test	Histology	1	2	4	9	0	n.a.	16	0	n.a.
	Cytology	0	2	4	13	0	n.a.	19	31	n.a.
Histological type	NSCLC	1	2	8	15	2		28	0	
	SCLC	0	2	0	7	1	0.585	10	0	n.a.
***mSHOX2* status ***	*mSHOX2+*	1	4	7	20	3		35	9	
	mSHOX2-	0	0	1	2	0	>0.999	3	22	<0.0001
Smoking status**	Non- smokers	0	1	2	0	0		3	13	
	Smokers	1	3	6	18	2	0.379	30	14	0.0013
	Former smokers	0	0	0	4	1		5	4	

Abbreviations: n.a. not applicable; LimDis – limited disease of SCLC, linked to Stage II; ExDis – extended disease of SCLC, linked to Stage IV. Due to low counts in these categories, statistical analysis in tumor samples was performed on 2×2 contingency tables for pooled data (stages I+II and III+IV). NSCLC – non-small cell lung cancer, SCLC – small cell lung cancer, mSHOX2+/- – positive/negative methylation of SHOX2.

**Sample was considered positive for SHOX2 methylation if positivity in at least one type of material was detected*

*** Analysis was performed on non-smokers vs. smokers or former smokers.*

collected from each patient: bronchial lavage and peripheral blood. Taken together we analyzed 138 samples in duplex real-time PCR reactions in parallels of plasma- and lavage-isolated DNA. The overall frequency of *SHOX2* methylation in analyzed set was estimated at 63.8% (44 out of 69).

Methylation of *SHOX2* gene was detected in 26 samples, specifically in 20 tumor samples and 6 negative samples. Unmethylated samples were represented by 33 specimens, 11 samples in group with diagnosed tumor and 22 in negative group. Results from 10 samples were considered invalid (Table 2). Out of 38 histologically positive patients, the analysis of *SHOX2* was valid in 37 bronchial lavage samples and 31 blood samples. The *SHOX2* methylation was detected in 31 of 37 valid bronchial lavage samples and 20 of 31 blood samples. On the other hand, the *SHOX2* was found unmethylated in 22 of 26 valid bronchial lavage samples from cancer-free patients and in 22 of 28 valid blood samples.

Presence of methylation in positive and negative group. Four different histological subtypes of lung cancer were distinguished in the positive sample group: squamous cell carcinomas, adenocarcinomas, small cell carcinomas, and large cell carcinomas. *SHOX2* methylation was detected in almost all cancer samples, however 3 adenocarcinomas were negative (Table 3).

For 31 patients of no-malignancy (negative) group, the following diagnoses were determined: undetermined diagnosis (n=6), bronchopneumonia (n=7), sarcoidosis (n=7), lymphomas (n=4), tuberculosis (n=3), mesothelioma (n=1), cancer metastasis of non-lung origin (n=1), pulmonary nodule (n=1), foreign body (n=1). The highest percentage of *SHOX2* methylated samples was found among lymphomas (50%). Also, in sample of mesothelioma, non-lung tumor metastasis and pulmonary nodule (n=3), *SHOX2* methylation was detected. On the other hand, methylation was not detected in any of TBC samples, and only in 14.3% of bronchopneumonia and also sarcoidosis samples.

Detailed pairwise comparison of patients, in which analysis from both types of material was valid, revealed that in the histologically positive group the result of *SHOX2* test correlated in 17 samples and was discordant in 13 samples. In the histologically negative group, the results of both tests correlated in 16 cases and were discordant in 7 cases. No significant

Table 2. Results of the SHOX2 methylation analysis linked to the type of the analyzed material.

Tumor samples (n = 38)	**Blood m*SHOX2* INVALID**	**Blood m*SHOX2*-**	**Blood m*SHOX2*+**
Lavage m*SHOX2* INVALID	0	0	1
Lavage m*SHOX2*-	2	1	3
Lavage m*SHOX2*+	5	10	16
Non-tumor samples (n = 31)	**Blood m*SHOX2* INVALID**	**Blood m*SHOX2*-**	**Blood m*SHOX2*+**
Lavage m*SHOX2* INVALID	0	4	1
Lavage m*SHOX2*-	3	15	4
Lavage m*SHOX2*+	0	3	1

Subjects are stratified according to the clinical status. Data within each stratum are paired according to the subjects and classified into the categories based on the type of sample and methylations status. Agreement and disagreement analysis of methylation is in detail presented within the Results section.

Table 3. Histological subtypes of positive samples divided according to the type of carcinoma.

Samples	NSCLC			SCLC	Total
	SCC	ADC #	LCC		
***mSHOX2* +**	19	6	1	9	**35**
% of row	54.29%	17.14%	2.86%	25.71%	
% of col	100%	66.67%	100%	100%	92.11%
***mSHOX2* -**	0	3	0	0	**3**
% of row	0%	100%	0%	0%	
% of col	0%	33.33%	0%	0%	7.89%
Total	19	9	1	9	**38**
% of n	50%	23.68%	2.63%	23.68%	

 # P= 0.0381 (Fisher-Freeman-Halton exact test)

Abbreviations: NSCLC – non-small cell lung cancer, SCC – squamous cell carcinoma, ADC – adenocarcinoma, LCC – large cell carcinoma, SCLC – small cell lung cancer.

**sample was considered positive for SHOX2 methylation if positivity in at least one type of material was detected*

asymmetry of disagreement in the subgroups was revealed. The overall agreement in a total of 53 samples was 62.26% (Kappa = 0.255; P = 0.029). Asymmetry of disagreement was not significant (P = 0.18).

Agreement analysis of methylation in different material. Agreement between definite diagnosis and either bronchial lavage or peripheral blood test on *SHOX2* methylation were above the lowest acceptable level of 70% of all classifications.

1. The bronchial lavage test results agreed in 84.13% (Kappa = 0.68, 0.49 to 0.86; P < 0.0001) with no significant asymmetry of disagreement (P = 0.53), PPV = 88.57% (73.26% to 96.8%), change = 30%, NPV = 78.57% (59.05% to 91.7%), change = 38%. Predictive value (PV) despite negative test was 21.43% (8.3% to 40.95%), change = -38%, sensitivity = 83.78% (67.99 to 93.81), and specificity = 84.62% (65.13 to 95.65).

2. The peripheral blood test results agreed in 71.19% (Kappa = 0.43, 0.20 to 0.65; P = 0.0004) with no significant asymmetry of disagreement (P = 0.22), PPV = 76.92% (56.35% to 91.03%), change = 24%, NPV = 66.67% (48.17% to 82.04%), change = 20%, PV despite negative test was 33.33% (17.96% to 51.83%), change = -20%, sensitivity = 64.52 (45.37 to 80.77), and specificity = 78.57 (59.05 to 91.71).

The independent contribution of each *SHOX2* methylation test to the prediction of malignancy was evaluated in multivariable models adjusted for age, gender, and smoking status (Table 4). ROC curve analysis was then used to define the optimal cut-off point for classification. In a ROC curve the sensitivity (probability of +ve result when malignancy is present) is plotted against 1-specificity (probability of +ve test when malignancy is absent). Resulting ROC curves for the models constructed using either peripheral blood test or bronchial lavage test were compared using the AUC criterion (the area under the curve). Since the prediction model involving bronchial lavage test results was not essentially better than that using the peripheral blood test results as seen from their AUC values, here we present the ROC curve for the latter model only (Figure 1).

Diagnostic performance of the model constructed with biomarker *SHOX2* methylation determined from the bronchial lavage samples at probability cut-off ≥ 0.5: sensitivity = 89.19%, specificity = 84.62%, PPV = 89.19%, NPV = 84.62%, PV despite negative test = 15.38%, correctly classified = 87.3%, likelihood ratio: LR of positive test = 5.797 (2.629 to 14.582), LR of negative test = 0.128 (0,050 to 0,299), AUC = 88.93%.

The model constructed with biomarker *SHOX2* methylation determined from the peripheral blood samples yielded quite similar results: sensitivity = 80.65%, specificity = 78.57%, PPV = 80.65%, NPV = 78.57%, PV despite negative test = 21.43%, correctly classified = 79.66%, likelihood ratio: LR of positive test = 3.763 (1.966 to 8.024), LR negative test = 0.246334 (0.115 to 0.483), AUC = 86.81% (Figure 1).

Table 4. Logistic regression models fitted on patients' data.

Model	Deviance (goodness of fit) P-value	Deviance (likelihood ratio) P-value	Coefficient	OR	95% CI	P-value
Lavage test	**0.6500**	**< 0.0001**	n.a.	n.a.	n.a.	n.a.
Intercept	n.a.	n.a.	-4.833	n.a.	n.a.	P = 0.0338
Gender	n.a.	n.a.	-1.262	0.283	(0.045 to 1.779)	P = 0.1784
Age	n.a.	n.a.	0.038	1.039	(0.976 to 1.105)	P = 0.2313
Smoking	n.a.	n.a.	2.677	14.553	(1.084 to 195.300)	P = 0.0433
m*SHOX2*	n.a.	n.a.	3.011	20.314	(4.125 to 100.000)	P = 0.0002
Blood test	**0.7800**	**< 0.0001**	n.a.	n.a.	n.a.	n.a.
Intercept	n.a.	n.a.	-6.387	n.a.	n.a.	P = 0.0028
Gender	n.a.	n.a.	-0.543	0.581	(0.112 to 3.001)	P = 0.5200
Age	n.a.	n.a.	0.052	1.054	(0.998 to 1.113)	P = 0.0603
Smoking	n.a.	n.a.	3.281	26.595	(2.595 to 272.600)	P = 0.0057
m*SHOX2*	n.a.	n.a.	2.396	10.979	(1.999 to 60.300)	P = 0.0058

Discussion

Alterations in DNA methylation are currently one of the most promising biomarkers in clinical research and thus *SHOX2* methylation may represent the potential improvement in lung cancer management. Expression of this gene is present in various tumor types, such as neuroblastomas and breast cancer [24].

The overall frequency of *SHOX2* methylation in our set was estimated at 63.8%, however it depends on the proportion of positive and negative samples in the set. Schmidt et al. [24] reported *SHOX2* methylation in 67.6% of analyzed samples. In the set of bronchial lavages the partial frequency of *SHOX2* methylation was estimated at the level 57.4%.

The average age of negative patients (54.1 years) and positive patients (66.7 years) reflects the expectation that the non-malignant samples were collected from younger patients than the positive ones. This finding confirms the fact that malignancies are usually associated with older age (Table 1). To accommodate this difference in age distribution the final model was adjusted for age (Table 4).

The smoking status of analyzed group was also monitored and higher percentage of non-smokers in the negative group than in positive group confirms expected strong and significant association between the presence of cancer and smoking status (Table 1). Due to this fact the models were adjusted to smoking status (Table 4).

Methylation of *SHOX2* in tumor samples. In the group of samples with lung cancer confirmed by cytology/histology, 92% of patients were positive for presence of *SHOX2* methylation. Only 3 patients diagnosed with adenocarcinoma were without detectable *SHOX2* methylation in any of analyzed material (Table 3). Differences in the observed proportions of *mSHOX2* positive test results among patients with squamous carcinoma and adenocarcinoma are in agreement with findings by Kneip et al. [21] and Schmidt et al. [24]. However, our detection rates of methylation in the histological subtypes were higher in magnitude. We identified 100% *SHOX2* methylation in SCLC and 100% in squamous carcinoma specimens versus 67% in adenocarcinoma specimens (P=0.0381), whereas Schmidt et al. [24] reported 97% *SHOX2* methylation in SCLC and 82% in squamous carcinoma specimens versus only 47% in adenocarcinoma specimens, although, they do not provide an explanation for the difference in the observed proportions. Our overall detection rate of positive patients was 92% in comparison with 72% reported by Kneip et al. [21] and 68% reported by Schmidt et al. [24]. These results indicate that specific type of lung cancer (SCLC and NSCLC squamous type) may be preferentially associated with *SHOX2* methylation. The same phenomenon was also previously described by Schmidt et al. [24] and requires further investigation.

Methylation of *SHOX2* in non-tumor samples. Interestingly, in the negative samples group the *SHOX2* methylation samples was detected in 50% of lymphomas, and in 100% of mesothelioma, pulmonary nodule and metastases samples. This indicates the possibility of using *SHOX2* methylation marker in other cancer malignancies as well, which was reported previously by Ilse et al. [25] and Dietrich et al. [26].

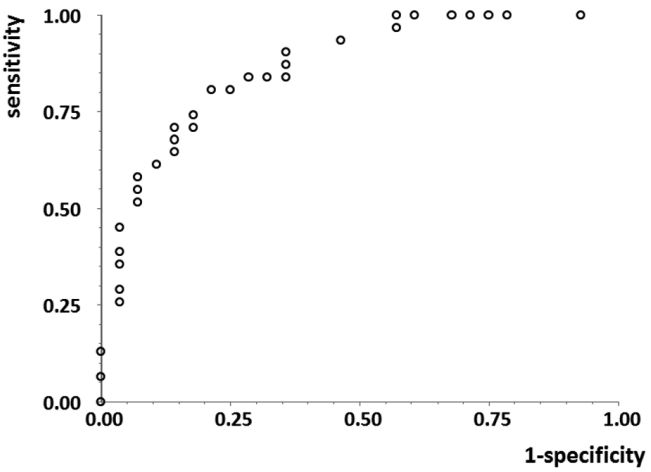

Figure 1. Receiver operating characteristic (ROC) curve for the patient series. Each point on the curve represents the multivariable model performance for a different cut-off probability threshold in deciding whether lung cancer is present.

Sensitivity and specificity of the test. Overall, 44 patients were detected as positive for *SHOX2* DNA methylation in at least one type of material, 9 of these samples were cytologically/histologically negative. On the other hand, 25 patients were *SHOX2* methylation negative and there was no lung tumor found in 3 of these samples. It is to speculate if these samples represented potentially false negative cases (Table 1).

These data translate into overall sensitivity of combined assays at 92% and overall specificity at 71%. Compared to other reported characteristics of *SHOX2* test sensitivities ranging from 60 to 81% and specificities from 78 to 96% [20, 21, 23, 25] our results are in general concordance. Above average sensitivity may be explained by use of two different materials in each patient.

If we look at each type of material separately, the sensitivity of the assay using bronchial lavage alone was 83.78% and specificity was 84.62%. Using the combined diagnostic model constructed from bronchial lavage assay, age, gender and smoking status increased sensitivity up to 89.19% with practically unchanged specificity. Classification performed with cut-off point of 0.5 was correct for 85.71% patients. Unlike lavage, blood plasma samples are collected non-invasively, thus representing a promising alternative. Similarly as with lavage samples, sensitivity and specificity was settled using logistic regression model at reasonable levels 80.65% and 78.57%, respectively, using same cut-off 0.5 and with 79.66% correctly classified patients. Kneip et al. [21] detected comparable sensitivity at 60% and somewhat higher specificity at 90%. These numbers were certainly affected by many factors such as

transportation of fresh blood, lower amounts of tumor DNA mainly in early stages of malignant disease as well as type of the normalization controls. Quite a few tests resulted in invalid analysis due to the insufficient amount of DNA isolated. All these issues need to be tuned before the assay can be used in clinics. However, we think that non-invasive character and relatively good sensitivity/specificity of this method at this point still make it a promising clinical choice.

The bronchial lavage showed higher levels of sensitivity and specificity of *SHOX2* methylation test compared to blood plasma. Thus we can conclude that performance of analysis at bronchial lavage samples represents the "gold standard" of lung cancer diagnosis using *SHOX2* methylation marker. However, the plasma samples may be a good alternative source of tumor DNA for *SHOX2* methylation analysis. Especially for patients who, for any reason, are unable to undergo bronchoscopy.

Agreement of methylation presence in different type of material. The *SHOX2* methylation was not correlating between sample types in 13 cytologically positive samples and 7 negative samples. Out of positive samples, 10 were lavage m*SHOX2* positive *(mSHOX2+)* and plasma m*SHOX2* negative (*mSHOX2-*). This may be explained by inappropriate sample transport, or insufficient amount of free tumor DNA in patient's plasma as discussed above. The case of 3 other samples which were lavage m*SHOX2-* and plasma m*SHOX2+* is little difficult to explain. One may speculate about insufficient number of tumor cells in lavage or presence of mucus or other contaminants, which may have affected the outcome of the lavage samples, or also presence of hidden malignant process.

In the group of negative samples, 4 were lavage m*SHOX2-* and plasma m*SHOX2+*. However, all of these samples came from patients with history of non-lung tumors. One of the patients was diagnosed with lymphoma in the past; others had metastasis of liver cancer, mesothelioma and sarcoidosis respectively. These findings may indicate that in most of these samples the presence of other type of cancer was reported and thus *SHOX2* may represent a promising marker for other malignancies as well. Again, the negative cytology in the group with 3 lavage m*SHOX2+*/plasma m*SHOX2-* samples is harder to explain. However, raised levels of oncomarkers in 2 of these patients may indicate the presence of so far undiscovered malignant process. The last patient denied the surgery therapy and a pulmonary nodule of unknown origin was diagnosed by x-ray.

In conclusion, we have confirmed that the methylation analysis of *SHOX2* gene using bronchial lavage samples represents a reliable test which can be used as an additional marker for lung cancer diagnosis in patients with negative cytology, who are positive by imaging methods or who have peripherally located lung lesions. The blood plasma test may represent usable alternative in cases when no bronchial lavage is available. Interestingly, if both types of materials were collected and analyzed from the patient, more tumors would be identified, as suggested by specificity of the combined approach. Further, the blood plasma test may be used for clinical decision based on a multivariable model where a diagnostic rule is created from multiple tests.

Finally, our results suggest that *SHOX2* methylation may be a potential marker for other malignancies. However, further analyses and validations are needed in order to identify these types of malignancies and the value of this test for them.

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