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Original paper

Cytological examination of pleural cavity lavage accompanied by the study of gene promoter hypermethylation of *p16* and *O6-methylguanine-DNA-methyltransferase* genes in diagnostics of non-small cell lung cancer metastatic changes into pleura

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Aim of the study: Metastases of non-small cell lung cancer (NSCLC) into pleura disqualify a patient from surgery and present a bad prognostic index. The aim of the study was to find out whether washing out the pleural cavity in such cases and examining obtained washings for presence of cancer cells will help to detect early NSCLC metastases into pleura, and also whether negative results of the cytology determine whether hypermethylation of these genes will increase the sensitivity of this examination.

Material and methods: The study consisted of the examination of 76 patients, including 59 operated on for NSCLC and 17 operated on for other reasons. Pleural washing fluid collected during the surgery was subjected to cytological examination as well as examined to determine the presence of promoter region hypermethylation of *p16* and *MGMT* genes.

Results: Positive cytological results of pleural lavage were confirmed in 4 persons (7%) with NSCLC. The presence of promoter region hypermethylation of one or both examined genes was found in 3 patients (18%) in the control group and in 47 (80%) in the study group. Sex, occupational exposure, smoking cigarettes, and NSCLC histological type did not have an influence on the presence of cancer cells or hypermethylation in the pleural lavage fluid. Positive cytology results were more frequent at the T4 stage of NSCLC. Hypermethylation was more frequent in the research group ($p < 0.01$). Cancer cells and hypermethylation did not occur more frequently in pleural lavage fluid of patients with metastases into pleura.

Conclusions: The cytological examination and promoter region hypermethylation assessment of the *p16* gene and *MGMT* gene in pleural lavage cells do not allow one to detect early metastasis of NSCLC into pleura.

Key words: lung cancer, pleura, cytology, DNA methylation, *p16* gene, O(6)-methylguanine-DNA methyltransferase.

Introduction

The multi-step concept of lung cancer carcinogenesis involves, apart from genetic disorders, also epigenetic disorders based on long-term changes of gene expression resulting from chromatin structure modification without changes in the DNA sequence. Two main epigenetic mechanisms include a modification of gene expression by CpG dinucleotides methylation and a change of chromatin structure induced by changes within histone proteins. Methylation of CpG dinucleotides localized on 5' ends in gene promoter regions, called "CpG islands", plays an important role in the process of their regulation. Hypermethylation of CpG islands causes damping of their expression and it takes place in the process of oncogenesis within suppressor genes. However, hypomethylation (demethylation) of the regions methylated normally causes an increase of gene expression, which can also favour neoplastic processes if it concerns protooncogenes. A high frequency of hypermethylation of gene promoter regions was shown in lung cancers as well as a high correlation between the presence of those disorders in tumour cells and sputum cells or in bronchial washings [1–3]. The presence of hypermethylation of the *p16* gene and *MGMT* gene was found in sputum cells 5 to 35 months before the appearance of any clinical symptoms and a diagnosis of lung cancer.

The *p16* gene (*INK4a, CDKN2A, ARF, MTS1*) is a gene coding proteins p16 and p19ARF, while the *MGMT* gene (*O6-methylguanine-DNA methyltransferase* gene) is a reparatory gene. Methylation analysis is a very promising marker used for early diagnostics of lung cancer development in persons exposed to such factors as tobacco smoke or uranium dust [4]. It might also provide a possibility of early diagnosis of lung cancer metastases into pleura, which occurs in its course very often. Their occurrence changes the patient's prognosis very significantly. The aim of this study is to determine how the examination of pleural washings accompanied by cell cytology and with the presence of hypermethylation of the selected genes can enable discovery of early metastases of lung cancer into pleura.

Material and methods

The study involved 76 patients (17 women, 59 men) aged 19 to 77 years (mean age 59 ±10 years) operated on in the Clinic of Thoracic Surgery in Zabrze, Silesian Medical University in Katowice.

The patients were divided into four groups:

- control group – 17 patients (12 women, 16 men), aged 19 to 75 years (mean age 52 ±10 years) without diagnosed lung cancer or fluid in the pleural cavity, operated on due to non-neoplastic reasons: oesophageal hiatus, oedema, emphysematous blebs, pneumoconiotic tumour, abscess, tuberculoma, inflammatory infiltration);
- study group I – 31 patients (8 women, 23 men), aged 48 to 77 years (mean age 61 ±9 years) with diagnosed NSCLC (non-small cell lung cancer) without features of infiltration into pleura and without fluid in the pleural cavity;
- study group II – 17 patients (2 women, 15 men), aged 50 to 73 years (mean age 62 ±6 years) with diagnosed NSCLC with pleura occupied by a neoplastic process (parietal pleura without neoplastic foci) without fluid in the pleural cavity;
- study group III – 11 patients (6 women, 5 men), aged 48 to 72 years (mean age 60 ±7 years) with diagnosed NSCLC with parietal pleura occupied by a neoplastic process, without fluid in the pleural cavity.

Inclusion criteria:

- patient's written consent for the surgery and genetic examinations;
- age over 18 years;
- no other coexisting neoplastic disease;
- no inductive chemotherapy or radiotherapy.

All the patients were in general good condition, i.e. 34 persons (45%) at level 1 according to the ECOG score and 42 (55%) at level 0. The patients' profile is presented in Table 1.

Material for the study was obtained during open lung surgery. During the operation, 250 ml of physiological saline was poured into the pleural cavity on the operated lung side. Next, the fluid was distributed with delicate movements of the chest around the pleural cavity, then the washings were collected into a paraffined, aseptic bottle, secured and sent for examinations. The further operation was carried on according to the primary plan. The study involved the evaluation of pleura in relation to the presence of neoplastic infiltration.

20 ml of washings were used for cytological examination. They were centrifuged, fixed with 95% ethanol and stained with haematoxylin and eosin. Then material were dehydrated in 95% ethanol, cleared using xylene and mounted with PDX. The assessment was made by two pathomorphologists. The results of cytological examinations were divided into three groups: positive, negative, and questionable (suspected of neoplastic features).

The rest of the pleural washings were centrifuged, then buffered with PBS fluid and centrifuged again. The obtained cellular sediment was frozen at –70°C or DNA was isolated immediately. The isolation was made with the set of MasterPureTM DNA Purification Kit from Epicentre Biotechnologies. Evaluation of the p16 gene and MGMT methylation status was done with the procedure described by Herman *et al*. [3]. 1 µ of genomic DNA in the volume 50 µl was denatured with 0.2 M NaOH for 10 min at 37°C. 30 µl of 10 mM hydroquinone solution (Sigma) and 520 µl of 3 M $NaHSO_3$ solution (Sigma) were added (both solutions were freshly made). Then they were mixed and incubated under a layer of mineral oil at 50°C for 16 hours. To purify such modified DNA, purifying resin Wizard DNA (Promega) was used and then the material was diluted in 50 µl of water. DNA modification was finished by adding NaOH to the final concentration of 0.3 M for 5 min at room temperature and by ethanol precipitation. After diluting in water, the solution was instantly used or stored at –20°C. The modified DNA was amplified for the *p16* gene by specific primers for the unmethylated sequence: forward, 5'-TTATTAGAGGGTGGGGCG GATCGC-3'; reverse, 5'-GACCCCAACCGCGACCGTAA-3' and by

Table 1. Patients' profile

Parameter		Control group	Study groups		
			I	II	III
number		17	31	17	11
grade	I (%)	–	22 (71)	10 (59)	0 (0)
	II (%)	–	6 (19)	2 (12)	6 (55)
	III (%)	–	3 (10)	5 (29)	5 (45)
ECOG Performance Status	0 (%)	11 (65)	12 (39)	8 (47)	5 (45)
	1 (%)	6 (35)	19 (61)	9 (53)	6 (55)
occupational exposure (%)		5 (29)	12 (39)	4 (24)	2 (18)
cigarettes	present smokers (%)	10 (59)	17 (55)	6 (35)	6 (55)
	former smokers (%)	2 (12)	10 (32)	7 (41)	5 (45)
	never smokers (%)	5 (29)	4 (13)	4 (24)	0 (0)
number of pack-years (mean)		5–60 (30)	20–104 (33)	16–80 (28)	20–52 (32)
mean amount of isolated DNA ng/µl		274	268	278	246
histopathological type of NSCLC	squamous cell (%)	–	15 (48)	8 (47)	5 (45)
	adenocarcinoma (%)	–	12 (39)	9 (53)	6 (55)
	large cell (%)	–	4 (13)	0 (0)	0 (0)

specific primers for the methylated sequence: forward, 5'-TTATTAGAGGGTGGGGTGGATTGT-3'; reverse, 5'-CCACCTAAATCAACCTCCAACCA-3'. The *MGMT* gene specific primers for the unmethylated sequence were: forward, 5'-TTTCGACGTTCGTTCGTAGGTTTTCGC-3'; reverse, 5'-GCACTCTTCCGAAAACGAAACG-3' and for the methylated sequence: forward, 5'-TTTGTGTTTTGATGTTTGTAGGTTTTTGT-3'; reverse, 5' AACTCCACACTCTTCCAAAAACAAAACA-3'. The PCR reaction mixture contained: 1 × PCR buffer (16.6 mM ammonium sulfate / 67 mM Tris, pH 8.8 / 6.7 mM Mgcl / 10 mM 2-mercaptoethanol), dNTPs (each of 1.12 mM concentration), primers (each 300 ng per reaction), modified DNA (approx. 50 ng) or unmodified DNA (50–100 ng) in the final volume 50 µl. The products of the PCR reaction were digested by adding 10 units of BstUI enzyme (New England Biolabs) for 4 hours. The next stage included ethanol precipitation and final gel analysis. The presence of hypermethylation of the studied genes was defined in the Laboratory of the Clinic of Internal Medicine and Diabetology in Zabrze.

The results were analysed with the Statistica program. Non-parametric Chi square test, Chi square with Yates' correction, V square test, Fisher's exact test and Mann-Whitney U test were used. Statistically significant results were assumed for $p < 0.05$.

The study was granted consent from the Bioethical Commission of the Silesian Medical University in Katowice (L.dz.NN-6501-72/II/05).

Results

In 4 patients, 3 men and 1 woman, the presence of neoplastic cells was found in pleural washings. Positive cytology results were found in 4 patients younger than 65 years. Cigarette smoking and occupational exposure did not significantly influence the frequency of positive or questionable cytology results of pleural washings (Table 2). It was not proved that histopathological type of NSCLC significantly influenced the frequency of positive cytology results of pleural washings. Significantly more frequent occurrence of cancer cells and cells suspected of neoplastic character of T4 stage than T3 stage ($p < 0.01$), T2 ($p < 0.01$) and T1 ($p = 0.038$) was found (Fisher's exact test was used). No statistically significant differences were found between the degree of lymph node occupation by neoplastic process and cytology results of pleural washings. NSCLC cells were present in pleural washings of 3% of patients at grade I, in 7% at grade II and 15% at grade III. Suspected cells were reported respectively in 9% of patients at grade I, 14% at II and 25% at III. In the control group no neoplastic cells were found in pleural washings. In the groups with NSCLC, no significant differences were found in occurrence frequency of neoplastic cells and/or suspected cells in relation to the infiltration found above the pleura.

The average amount of isolated DNA in pleural washings where neoplastic cells were found was 370 ng/µl, and in the washings with the cells suspected of such character 332 ng/µl. In cases of negative cytology results, the average amount of DNA was lower – 233 ng/µl. It was not proved that neoplastic or suspected cells occurred more frequently in pleural washings with higher amounts of isolated DNA.

The presence of promoter region hypermethylation of one or both studied genes was found in 3 patients (18%) in the control group and in 47 (80%) in the study groups. Promoter region hypermethylation of only one gene was noted only in one case (6%) in the control (it was the *MGMT* gene) and in 11 cases (19%) in the study groups. Presence of hypermethylation of one or both studied genes was found in 13 women (76% of all women). Among men, 37 (63%) had excessive methylation of one or both genes. The presence of *p16* gene hypermethylation was found in 34 patients aged under 65 years and in 11 older patients. Excessive promoter region methylation of the *MGMT* gene was recorded in 34 persons (64%) aged under 65 years and in 9 (39%) aged 65 years or more. 15 patients out of 23 with occupation exposure had hypermethylation of one or both genes. The number of smoked cigarettes did not influence the increase of hypermethylation frequency of the studied genes.

Histological type of cancer did not have a significant influence on frequency of hypermethylation occurrence. In adenocarcinoma, the gene p16 was excessively methylated in 22 patients, the *MGMT* gene in 21. Patients with squamous cell carcinoma had a insignificantly smaller amount of hypermethylated promoter regions of those genes (19 persons p16 and 17 persons MGMT). In 2 out of 4 patients who had a diagnosis of large cell carcinoma, hypermethylation of both genes was observed. *p16* gene hypermethylation was noted in 9 cases at stage T1, in 17 at stage T2, in 13 at T3 and in 4 at T4. The *MGMT* gene was excessively methylated in 9 persons at stage T1, in 15 at T2, in 12 at T3 and in 4 at T4. No significant differences were found in frequency of promoter region hypermethylation of the *p16* gene or *MGMT* gene in relation to the T and N status. However, significantly more frequent hypermethylation of one or both genes in the pleural washing cells was observed at stage T3 than at stage T4 ($p = 0.026$ Fisher's exact test). Frequency of hypermethylation occurrence at individual stages of NSCLC

Table 2. Results of pleural lavage in groups

	Parameter	Control group	Study groups		
			I	II	III
cytological examination	positive (%)	0 (0)	1 (3)	2 (12)	1 (9)
questionable (suspected) (%)	1 (6)	6 (19)	1 (6)	1 (9)	
negative (%)	16 (94)	24 (77)	14 (82)	9 (82)	
hypermethylation	both genes (%)	2 (12)	18 (58)	12 (71)	6 (55)
	at least one gene (%)	3 (18)	24 (77)	14 (82)	9 (82)
	P16 gene (%)	2 (12)	22 (71)	13 (76)	8 (73)
	MGMT gene (%)	3 (18)	20 (65)	13 (76)	7 (64)

Table 3. Results of pleural lavage depending on histopathological type and grade of NSCLC

		Hypermethylation			
		p16		MGMT	
		+	–	+	–
histopathological type of NSCLC	adenocarcinoma (%) squamous cell (%) large cell (%)	22 (81) 19 (68) 2 (50)	5 (19) 9 (32) 2 (50)	21 (78) 17 (61) 2 (50)	6 (22) 11 (39) 2 (50)
grade	I (%) II (%) III (%)	21 (66) 12 (86) 10 (77)	11 (34) 2 (14) 3 (23)	20 (63) 11 (79) 9 (69)	12 (38) 3 (21) 4 (31)
T	1 (%) 2 (%) 3 (%) 4 (%)	9 (82) 17 (63) 13 (93) 4 (57)	2 (18) 10 (37) 1 (7) 3 (43)	9 (82) 15 (56) 12 (86) 4 (57)	2 (18) 12 (44) 2 (14) 3 (43)
N	0 (%) 1 (%) 2 (%)	34 (71) 6 (75) 3 (100)	14 (29) 2 (25) 0 (0)	32 (67) 6 (75) 2 (67)	16 (33) 2 (25) 1 (33)

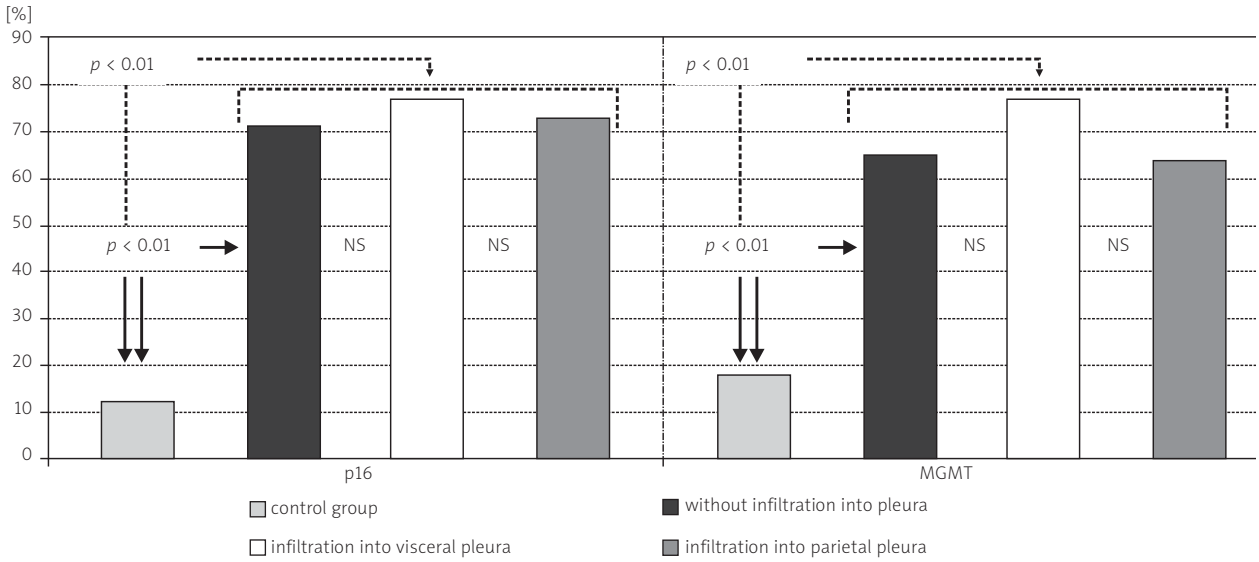

Fig. 1. Hypermethylation of selected genes in control and study groups

is presented in Table 3. Statistical analysis did not show any significant differences in the occurrence of promoter region hypermethylation of the studied genes in relation to NSCLC grade.

In the control group, hypermethylation of both genes was found in 2 persons and of the *MGMT* gene only in 1 person. Among patients with NSCLC, both genes had incorrect promoter region methylation in 36 patients, only the *p16* gene in 7, and only the *MGMT* gene in 4. Significantly less common occurrence of excessive promoter region methylation of the studied genes was found in the control group than in other study groups ($p < 0.01$ µ2 test with Yates' correction). No significant differences in occurrence frequency among groups with NSCLC were found. The results are presented in Fig. 1.

The average amount of isolated DNA in pleural washings where no promoter region hypermethylation of the selected genes was found was 280 ng/µl. In cases of excessive promoter region methylation of the *MGMT* gene, it was 230 ng/µl, and of the *p16* gene it was 231 ng/µl. It was determined that higher amounts of isolated DNA in pleural washings had an influence on evaluation results of the presence of promoter region hypermethylation of the studied genes. In 3 cases out of 4 (75%) pleural washings where the presence of neoplastic cells was observed, the presence of promoter region hypermethylation of both genes was also found. Among patients with questionable cytology results, 6 out of 9 patients (67%) had excessive methylation of both genes. In the remaining cases of positive or questionable cytology results, no presence of hypermethylation was noted. In cases of pleural washings with negative cytology results, 36 patients out of 63 (57%) had promoter region hypermethylation of the *p16* gene, and 34 (54%) of the *MGMT* gene. It was not found that excessive methylation of the studied genes in pleural washings where neoplastic cells or suspected cells were discovered occurred more often.

Discussion

Evaluation of the usefulness of cytological examination of pleural washings in patients with lung cancer has been a subject of numerous publications. Neoplastic cells in

pleural washings were discovered in 3.2–46% of patients [5, 6]. Frequency of positive cytology results of pleural washings in our study was 7% and it is similar to the results presented by Hillerdal (12.3%), Kondo (9%) and Higashiyama [5, 7, 8]. No factors such as gender, age, occupational exposure or cigarette smoking were found to have influenced the frequency of positive cytology results of pleural washings. Similar observations were also made by Satoh and other authors [9, 10].

Like Enastu and Vicidomini, we have not found that the histopathological type of NSCLC favoured more frequent occurrence of neoplastic cells in pleural washings [10, 11]. Other authors observed significantly more frequent occurrence of positive cytology results in washings in adenocarcinoma [7, 9, 12, 13]. Meinoshin Okumura *et al*. found more frequent occurrence of neoplastic cells in pleural washings together with an increase of T stage [14]. Kjellberga *et al*. did not confirm those observations [15]. We also did not find, similarly to Okada and other authors, relations between positive cytology results of pleural washing and stage of lymph node involvement (N status) [6, 7, 11, 13, 15]. Eagan *et al*., similarly to Satoh and Enatsu, found that neoplastic cells occur more often in patients with greater involvement of lymph nodes by the neoplastic process [9, 10, 12]. It is thought that the frequency of neoplastic cells' occurrence in pleura depends on the stage of development of the lung cancer [5–7, 9, 11, 12, 14, 16]. Such a relation, similarly to our results, was not observed by Kjellberg. He also found no relation between the grade of neoplastic cell differentiation in lung cancer and cytology results of pleural washings [15]. The frequency of neoplastic cells' occurrence in pleural washings is influenced by pleura occupation by the neoplastic process, especially in parietal pleura [5, 7, 9, 12–14]. Vicidomini recorded more frequent occurrence of neoplastic cells in pleural washings at the moment of appearance of neoplastic infiltration into the pleura. However, he did not observe a significant difference in cytology results of washings in the patients with occupied parietal pleura in comparison to occupied pulmonary pleura [11]. Similarly to Kjellberg *et al*., we did not confirm in our study significantly more frequent occurrence of neoplastic cells in the washings in patients with pleura occupied by a neoplastic process [15].

The presence of epigenetic changes, including incorrect promoter region methylation of p16 and *MGMT* genes in neoplastic cells of lung cancer, was found with variable frequency. In adenocarcinomas, this irregularity in the *p16* gene was observed in 83% of patients, and in the *MGMT* gene in 17% of patients [17]. Hypermethylation with all types of NSCLC occurred in the *p16* gene in 67% of patients, in the *MGMT* gene in 62% [18]. Other authors described the occurrence of this disorder in patients with lung cancer from 15.4% for the *p16* gene and 10% to 18.7% for the *MGMT* gene [19, 20].

Ng *et al*. analyzed the *p16* gene in 33 patients with NSCLC. No control group was created. Pleural washings, serum and removed tumour were used in the study. The washings were taken twice, before lobectomy or pneumonectomy and immediately afterwards. Abnormal methylation in the cancer was found in 14 patients (42%), in serum in 2 patients (6%). The frequency of *p16* gene hypermethylation in pleural washings was lower than in our results. In the first rinsing, it was recorded in 3 cases (9%), in the second one in 4 cases (12%). The patients who had no hypermethylation found in the neoplastic tumour did not have it in washing cells or serum either [21].

In one patient from the control group in our study, we found promoter region hypermethylation of the *p16* gene and the *MGMT* gene. In one person from this group, hypermethylation of only *MGMT* gene was found. Both patients were re-examined, being suspected of neoplastic disease. Their two-year observation and additional examinations, including chest CT, did not confirm the presence of malignant cancer. In the literature cases of excessive promoter region methylation in patients without cancer are reported with variable frequency.

In our study, patient's gender did not significantly influence the promoter region hypermethylation of the studied genes in the pleural washing cells. The same situation referred to age in cases of the *p16* gene; however, MGMT was excessively methylated more often in persons under 65 years old. Other authors did not find out whether those factors had any influence on methylation in the patients with NSCLC [19, 22–24].

Among patients without lung cancer more frequent occurrence of hypermethylation of the *p16* gene and *MGMT* gene were found in elderly people. A considerable majority of patients with the confirmed exposure to dust and gases worked underground in a coal mine. Occupational exposure did not influence the frequency of the observed hypermethylation of the studied genes. Such a relationship was observed by Divine. The patients that he examined were exposed to ionizing radiation in a uranium mine [24]. Furthermore, cigarette smoking did not significantly influence the hypermethylation appearing in pleural washing cells in our study. More frequent occurrence of hypermethylation in smokers in NSCLC was also not observed by other authors [19, 24–26]. A positive correlation between cigarette addiction and hypermethylation of p16 and *MGMT* genes in NSCLC cells was found by Liu *et al*. [27].

Frequency of hypermethylation of the studied genes and cancer stage (T, N) were similar in individual histopathological types of NSCLC in our study. A dependence between histopathological type and hypermethylation presence was also not found in other studies [19, 21, 27–29]. Similar results were also obtained by other authors [18, 27–29]. Ng *et al*. did not find that the presence of *p16* gene promoter region hypermethylation in the pleural washing cells was related to neoplasm grade; however, he observed such a relation in neoplastic tumour and serum of those patients. Those authors found that excessive methylation in washing cells before lobectomy or pneumonectomy, similarly as in our study, did not depend on the degree of pleura involvement. Hypermethylation is found more frequently in the washings made after lobectomy or pneumonectomy in the patients with extensively occupied pleura [21].

Summary

On the basis of the obtained results and data from other authors it can be stated that pleural washings in patients with NSCLC made in order to obtain information on pleural cancer does not bring any answers. Positive results of pleural washing cytology are found very rarely, and the absence of neoplastic cells in those washings does not confirm

absolutely that the pleura is free from metastases. The presence of neoplastic cells in those washings does not indicate the cancer stage, which includes the T and N status. Improving pleural washing cytology by defining the presence of promoter region hypermethylation of p16 and *MGMT* genes in the washing cells does not increase the sensitivity of detection of early metastases into the pleura. Unlike cancer cells, which are rarely found in washing cytology, the presence of hypermethylation is observed quite often. It is recorded even in patients without cancer. This fact decreases this method's specificity. These results are not influenced by gender, age, cigarette smoking or occupational exposure. In conclusion:

1. Statistically significant relations between the presence of cancer cells in pleural washings and NSCLC histological type and stage and localization to the pleura were not found.

2. Statistically significant relations between the presence of promoter region hypermethylation of the selected genes in the pleural washing cells and NSCLC histological type, including localization to the pleura, were not found.

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