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ARTICLE

The significance and factors leading to heteroploidy in pleural lavage cytology

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Abstract

Introduction: Positive intraoperative pleural lavage cytology (pre-resection PLC) is considered a prognostic risk factor of primary lung cancer surgery. Positive findings suggest subclinical metastasis of malignant cells in the thoracic cavity. In this study, we attempted to determine the incidence of and risk factors for positive pleural lavage cytology (PLC) in primary lung cancer patients. **Methods:** We included 62 surgically treated patients who had been diagnosed with non-small-cell lung cancer and had their PLC status examined between November 2019 and November 2021. PLC was measured by the circulating tumour cell (CTC) method, and we searched for factors predictive of a PLC-positive status. **Results:** PLC (+) was identified in 22 of the 62 patients (35.5%) and was associated with air bronchogram (p=0.042), pathological classification (p=0.008) and tumour stage (p=0.017). There was no significant difference in other factors between PLC (+) and PLC (-) patients. Binary logistic regression analysis showed that the odds ratio of PLC positivity in the population with bronchial signs was 4.200 compared with the population without bronchial signs. **Conclusion:** Lung cancer patients with bronchial signs on imaging have a greater probability of PLC positivity. The probability of PLC positivity in patients with carcinoma in situ is reduced, and the probability of PLC positivity increases when the tumour metastasizes.
Abbreviations: NSCLC = non-small-cell lung cancer; PLC = pleural lavage cytology; CEA = carcinoembryonic antigen; EGFR = epidermal growth factor receptor

Keywords: air bronchogram; heteroploidy; non-small-cell lung cancer; pleural lavage cytology

1. INTRODUCTION

Lung cancer is the most common cancer worldwide, and it is a leading cause of cancer-related death[1-3]. The 5-year survival rate of stage IA1 non-small-cell lung cancer (NSCLC) can reach 90%[4]; however, the 5-year survival rate of stage IV NSCLC is less than 10%[5]. In

China, the number of deaths from lung cancer is expected to exceed one million by 2025[6, 7].

According to the eighth edition of the TNM lung cancer classification, patients are expected to benefit from the resection of primary and metastatic lesions when lung cancer has oligometastatic foci[8]. However, when malignant pleural effusion or intrathoracic dissemination is present, the stage is regarded as pM1a, which indicates that the lesion is not suitable for radical resection. Therefore, we believe there is a time period of tumour progression during which the tumour spreads to the thorax but does not form malignant pleural effusion or spread further. Regarding current test methods, scattered micrometastases in the thoracic cavity of lung cancer is difficult to identify using conventional examination methods. CT[9] examination can only observe centimetre and millimetre layers, and changes at the cellular level cannot be observed. When the tumour does not invade the visceral pleura, pathological analysis can only be used to examine lesions in the lung tissue, not material obtained from the pleural cavity; however, PLC can fill this gap.

PLC was first implemented by Spjut[10] in 1958, Kondo et al.[11] found that PLC (+) status before surgery affected the survival rate of patients in 1989. PLC positivity is more common in advanced tumours[12-17]. In addition, other studies have suggested that pleural invasion (PI), serum carcinoembryonic antigen (CEA), adenocarcinoma, epidermal growth factor receptor (EGFR) mutation status, tumour diameter[18-20], tumour metastasis, vascular invasion and lymph node metastasis[15, 21, 22] are significantly related to PLC positivity.

In contrast to other studies, in this study, we used a method to identify heteroploid cells in pleural lavage fluid. The DNA content of normal human cells is mostly diploid, and gene loss, mutation, abnormal amplification, chromosome shift and fusion caused by carcinogenic factors are considered to be early molecular events of tumorigenesis, which can lead to an increase in the DNA content. The emergence of heteroploid cells in pleural lavage fluid highly is suggestive of the existence of malignant pleural effusion[23, 24]. Some scholars[25-28] believe that the appearance of abnormal diploid cells indicates early-stage carcinogenesis. According to the PLC status of NSCLC patients, we aimed to identify the factors related to a positive PLC status and the association with clinical and pathological features.

2. PATIENTS AND METHODS

After the operation, we uploaded the patients' information to the ResMan database. We retrospectively reviewed 77 surgically treated patients with primary lung cancer who underwent PLC examinations between November 2019 and November 2021 at the XXX University. Before the experiment, exclusion criteria were established, as follows: malignant pleural effusion; chemotherapy; radiotherapy; targeted therapy or immunotherapy; extensive intrathoracic adhesions found during the operation; a poor general condition, or poor function of important organs, such as the heart, liver and kidney; and participation in other clinical trials. However, for three patients with stage IV disease, pathological tissues could not be obtained through percutaneous lung puncture and tracheoscopy, so PLC examination was performed during the operation. We excluded 15 patients who were determined to have

benign lesions after surgery, so a total of 62 eligible patients were ultimately analysed.

Thus far, all procedures have been performed using video-assisted thoracoscopy (VATS). When there was no evidence of dissemination or pleural metastasis, before tumour resection, 500 ml of 37°C saline was infused onto the surface of the pleura near the tumour. Then, 500 ml of pleural lavage fluid was transferred to a bottle and sent to the pathology department for heteroploidy examination. The pleural lavage fluid was centrifuged at 1900 rpm for 5 minutes. After centrifugation, the red blood cells were lysed, the white blood cells and impurities were removed, and the cells were stained and detected by FISH. Finally, the presence of heteroploidy was observed by fluorescence microscopy (Figure 1 Figure 6). If heteroploidy was detected in the pleural lavage fluid, the patient was classified as PLC(+); otherwise, the patient was classified as PLC(-).

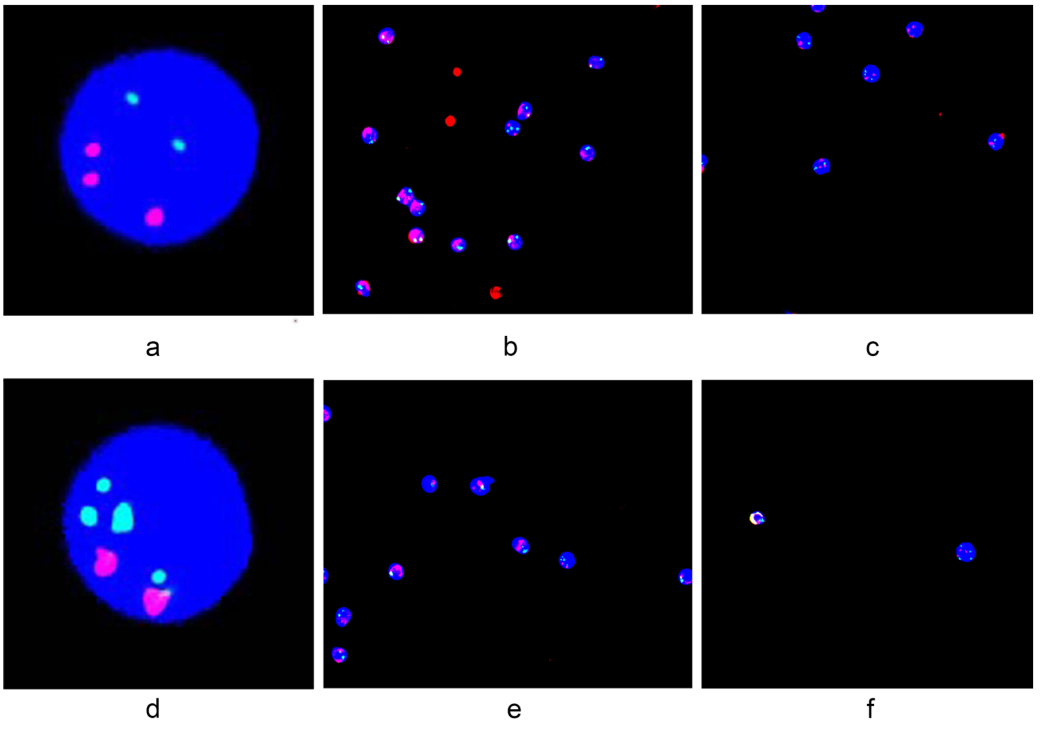

Figure 1. heteroploidy observed by fluorescence microscopy; a, b & c indicate triploidy; d, e & f indicate tetraploidy

2.1 Preoperative examination

Patients underwent lung function and routine blood biochemistry assessments, and routine clinical staging was performed before surgery with head and chest computed tomography scans, abdominal colour ultrasound and tumour indicators. If the index was abnormal, patients underwent PET-CT examination. Pulmonary function and arterial blood gas analyses were performed to determine whether the patient could tolerate surgery.

The imaging observation index was the double-blinded observation of CT images of all patients by two radiologists with middle- or senior-grade professional titles. When the two clinicians had different opinions in the evaluation of CT signs, they either reached a consensus or asked a higher-level expert to analyse and evaluate the images to reach a final conclusion. This study aimed to identify high-risk factors for PLC (+). Tumour classification was classified according to the WHO classification[29], and tumour staging was classified according to the 8th Edition of TNM staging.

2.2 Statistical analyses

The following clinicopathological features of the patients were extracted from the ResMan Database for comparisons of the PLC (+) group and the PLC (-) group: sex, age, tumour component, tumour location, whether there was an invasive operation performed before surgery, whether there was pleural traction on CT, whether the tumour had a vascular trajectory, whether the tumour had a cavity, whether the tumour had air bronchogram, degree of tumour infiltration, patient blood type, distance from the tumour to the pleura, distance from the tumour to the pleura/tumour size, whether there was a micropapillary component, tumour size, CEA value, lymph node metastasis (PN) and stage of the tumour.

The two groups of data were analysed by SPSS 25.0 statistical software. The measurement data conforming to a normal distribution are expressed as the mean ± standard deviation, and a two independent samples t test was used for comparisons between the two groups. The measurement data with a non-normal distribution are expressed as the median (interquartile interval), and the rank sum test was used for comparisons between the two groups. Count data are expressed as a percentage and compared with the 2 test or Fisher's test. $P < 0.05$ indicated a statistically significant difference. Binary logistic regression was used to analyse the risk factors for PLC.

3. RESULTS

A comparison of the clinicopathological features of the PLC(+) group and the PLC(-) group is shown in Table 1. PLC positivity was observed in 22 patients (35.5%), and PLC negativity was observed in 40 patients (64.5%). According to the chi-square test (Table 1), on CT imaging, there were significant differences in whether the tumour exhibited air bronchogram signs ($P = 0.042$), the degree of tumour infiltration ($P = 0.008$) and the stage of tumour ($P = 0.041$) ($P < 0.05$). There was no significant difference in PLC status based on sex, age, tumour component, tumour location, whether the patient underwent an invasive operation prior to surgery, whether there was pleural traction on CT, whether the tumour had a vascular trajectory, whether the tumour had a cavity, patient blood type, whether there was a micropapillary component, or lymph node metastasis (PN).

According to the independent sample t test, there was no significant difference between patients of different ages. According to the rank sum test, there was no significant difference between the distance from the tumour to the pleura, the distance from tumour to the pleura/tumour size, tumour size and CEA value and PLC status ($p > 0.05$) (Table 1).

Through binary logistic regression analysis, it can be seen that an air bronchogram pattern is an independent risk factor for PLC positivity ($p < 0.05$), and patients with air

Table 1. Single factor analysis.

	PLC(-)(N=40)	PLC(+)(N=22)	X^2/t/F	*p*
Sex			0.373	0.541
male	15(37.5%)	10(45.5%)		
female	25(62.5%)	12(54.5%)		
Age	49.38±9.36	48.82±8.89	0.228	0.820
Tumour location			/	0.244
upper left	9(22.5%)	6(27.3%)		
lower left	7(17.5%)	7(31.8%)		
upper right	11(27.5%)	5(22.7%)		
middle Right	0(0%)	1(4.5%)		
lower right	13(32.5%)	3(13.6%)		
Invasive operation			/	1.000
no	37(92.5%)	20(90.9%)		
yes	3(7.5%)	2(9.1%)		
Pleural traction			2.867	0.090
no	27(67.5%)	10(45.5%)		
yes	13(32.5%)	12(54.5%)		
Vascular trajectory			/	0.151
no	5(12.5%)	0(0%)		
yes	35(87.5%)	22(100%)		
Tumour cavity			0.658	0.417
no	27(67.5%)	17(77.3%)		
yes	13(32.5%)	5(22.7%)		
Air bronchogram			/	0.042
no	36(90%)	15(68.2%)		
yes	4(10%)	7(31.8%)		
Degree of tumour infiltration			/	0.008
carcinoma in situ	12(30%)	0(0%)		
microinvasive carcinoma	3(7.5%)	3(13.6%)		
infiltrating carcinoma	25(62.5%)	19(86.4%)		
Blood type			/	0.438
A type	10(25%)	6(27.3%)		
B type	12(30%)	3(13.6%)		
AB type	5(12.5%)	2(9.1%)		
O type	13(32.5%)	11(50%)		
Distance from the tumour to the pleura (mm)	8.95（2.5,15.5）	5.15（0,13.3）	-1.221	0.222
Distance from the tumour to the pleura/tumour size	1.16（0.1493846,2.71）	0.48（0,1.14）	-1.480	0.139
Micropapillary component			/	0.499
no	34（85%）	17（77.3%）		
yes	6（15%）	5（22.7%）		
Tumour size (mm)	9.5（6,14）	11.5（8,18）	-1.187	0.235
Lymph node metastasis			/	0.399
clinical N0	38（95%）	19（86.4%）		
clinical N2	2（5%）	2（9.1%）		
clinical N3	0（0%）	1（4.5%）		
CEA	1.775（1.315,2.55）	1.505（0.78,3.05）	-0.471	0.638
Tumour component			/	0.152
pure ground glass nodule	28(70%)	10(45.5%)		
subsolid nodule	5(12.5%)	5(22.7%)		
solid nodule	7(17.5%)	7(31.8%)		
Tumour stage (metastasis)			/	0.041
yes	40(100%)	19(86.4%)		
no	0(0%)	3(13.6%)		

bronchograms are more likely to have PLC than those without these bronchial signs. The odds ratio of PLC positivity in patients with air bronchograms was 4.200 compared with those without bronchial signs (Table 2), and the degree of tumour infiltration and tumour stage could not be analysed due to the presence of extreme values.

Table 2. Binary logistic regression.

	B	Standard error	Wald	freedom	Significance	Exp(B)
Air bronchogram	1.435	0.698	4.226	1	0.040	4.200
Constant	-0.875	0.307	8.115	1	0.004	0.417

4. DISCUSSION

In contrast to other studies, this study is the first to find evidence of heteroploid cells in pleural lavage fluid, to determine the incidence and risk factors for PLC in primary lung cancer and to explore the prognosis of these patients.

Heteroploidy is an objective marker for the diagnosis of cancer[30], and the sensitivity of heteroploidy for the diagnosis of malignant pleural effusion was 52% 94%[30], which means that its sensitivity is better than that of tumour cell identification. This may also have increased the positivity rate in this study. Some studies have also shown that the specificity of heteroploidy in the diagnosis of malignant pleural effusion is 70% - 91%[31], which also increases the possibility of false positives in the experimental data. Although one study[26] showed that heteroploidy can appear in some patients with benign tumours and inflammatory lesions, in this study, the PLC results of 15 patients who had benign tumours were negative, which also shows that this experiment has a strong specificity.

Heteroploidy is the most common cause of solid tumours[32]. Coward et al.[33] demonstrated that heteroploidy can promote the evolution of tumours and help them to acquire drug resistance capabilities. The presence of heteroploid cells in pleural lavage fluid does not mean that the patient definitely has metastasis and tumour cell dissemination in the thoracic cavity. The growth of tumours requires a suitable environment and opportunity. After being affected by carcinogens and tumour promoters, only a few precancerous cells have the ability to proliferate indefinitely; therefore, patients with PLC (+) should be treated more actively after surgery.

In most reports, the PLC positivity rate in patients with non-small-cell lung cancer was 3.0% - 18.2%[18, 19, 34]. The PLC (+) rate was 38.6%[35] in another study, while in this study, it was 35.5%. The high positivity rate observed in this study may be because the patients included all had adenocarcinoma. Some studies have reported that adenocarcinoma is more likely to cause malignant pleural effusion relative to other types of lung cancer[36, 37]; at the same time, it is also easy to develop PLC(+)[14, 19, 38-40]. The EGFR positivity rate in patients with adenocarcinoma is also increased, and patients with EGFR mutations are prone to both malignant pleural effusion[41] and PLC(+)[20].

In this study, we found that the presence of an air bronchogram pattern on CT imaging was highly correlated with PLC(+). The odds ratio of PLC (+) in patients with bronchial

signs was 4.2 compared to patients without bronchial signs. This result may be due to the direct connection of the distal bronchus with the visceral pleura[42]. The visceral pleura has a mechanical relationship with lung activity and promotes the patency of alveoli and bronchioles[43], and heteroploidy may fall into the pleural cavity along this gap. Interestingly, there was no significant difference between the PLC (+) group and the PLC (-) group when patients had lymph node metastasis, and patients with mutations in EGFR[44] may have a lower risk of lymph node metastasis than those with other types of lung cancer. The patients we included all had lung adenocarcinoma, and it is possible that tumour cells may enter the pleural cavity through the terminal bronchus earlier than lymph node metastasis, which needs further research.

In our work, according to the univariate analysis, we found that all patients with tumours that had metastasized were PLC positive, and for those with tumours in situ, they were all PLC negative. Thus, it can be considered that the stage of the tumour and the degree of tumour infiltration are independent risk factors. However, due to the high clinical value of these two factors, we cannot draw certain conclusions. When the pathological result indicates an in situ tumour, the tumour has no risk of distant metastasis. When distant metastasis is noted, although there may be no malignant pleural effusion in the chest, sub-metastasis may have developed.

Cytological staging of ovarian cancer has been included in the peritoneal lavage system, and malignant pleural effusion has been classified as pM1a in the eighth edition TNM staging for lung cancer. Before the development of malignant pleural effusion, PLC (+) is considered to be an indicator of the subclinical dissemination of malignant cells in the thoracic cavity. In 2010, the international PLC cooperation group recommended PLC as a routine examination for patients with non-small-cell lung cancer. PLC(+) is an independent risk factor for the postoperative survival rate, and the T stage of NSCLC patients with PLC(+) whose stage was I-III should be increased by one grade[12, 45]. Some studies[12, 45] have pointed out that local intrapleural therapy does not improve the survival rate of patients with PLC(+); therefore, adjuvant chemotherapy may be a treatment option[12, 46, 47]. By incorporating PLC status into TNM, we can identify more high-risk patients with stage I cancer who are not suitable for chemotherapy and provide them more tailored therapy. We also found that when the tumour is accompanied by air bronchogram signs on chest CT, the probability of heteroploidy in the pleural lavage fluid is increased, which should remind clinicians who manage patients with early NSCLC to use a more active treatment strategy or more rigorous detection methods after surgery.

5. Limitations

This study has some limitations. First, this was a single-centre retrospective study, and the sample size was relatively small. Second, we used the degree of air bronchogram signs as a predictor of PLC status, but in fact, different film readers may obtain different results, so subjective bias may exist. Third, the volume of saline used was changed during the study. There is no consensus on how much saline should be used, but this change may have affected the results. Fourth, EGFR mutation is a common mutation in Asia[48], which may have led to the high positive rate observed herein, so it may not be appropriate to extrapolate the

current findings to other ethnic groups. Large-sample RCT research is needed in the future to clarify the conclusion.

6. CONCLUSION

If the tumour shows air bronchogram signs on CT imaging or patients with adenocarcinoma are classified as having microinvasive cancer or invasive cancer, they will show a high rate of PLC(+) and should be carefully examined for pleural dissemination or effusion so that appropriate treatment can be provided to these patients after surgery. When patients have distant organ metastasis, the likelihood of PLC positivity will also increase, as will the risk of intrathoracic metastasis. For these patients, clinicians should pay close attention to whether there is pleural effusion in the later stage to improve their quality of life as much as possible.

Clinical trial registration number

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