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ORIGINAL ARTICLE

Application of malignant pleural effusion cell blocks in the diagnosis and personalized treatment of advanced non-small cell lung carcinoma*

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Abstract

Objective The aim of the study was to investigate the efficacy of immunocytochemistry and related gene detection using cell block for the diagnosis and individualized treatment of advanced lung cancer.

Methods Sixty-five malignant pleural effusion specimens were collected to make cell blocks, which were used for hematoxylin and eosin (H&E) staining, immunocytochemical studies, and gene sequencing of the tumors to guide the individualized diagnoses and treatment of the given tumors.

Results The tumor cells in the cell block sections were abundant in number with high quality cellular structures, and the histological morphological characteristics were partially maintained. Immunocytochemical staining was helpful in identifying the cell origin and tumor classification, and amplification refractory mutation system (ARMS) was used to determine the mutation status of epidermal growth factor receptor (EGFR). Of the 65 samples, 50 had a diagnosis of adenocarcinoma, 7 were pulmonary squamous cells, 6 were small cell carcinoma of the lung, and 2 were mesothelioma. The morphological features of the tumors were as follows: acinar formation, papillary and single cells for adenocarcinoma; intercellular bridges for squamous cell carcinoma; and morphology of the small cells is similar to that of the smear. Correlating with the results of immunocytochemical staining and clinical data analysis, 40 cases were confirmed as pulmonary adenocarcinoma, with an additional 4 cases of breast cancer, 3 cases of ovarian adenocarcinoma, and 3 cases of colorectal adenocarcinoma. Of the 47 non-small cell lung carcinoma (NSCLC) patients, EGFR mutations were detected in 26 cases (55.3%) by ARMS, with four mutation types: exon 19 deletion (13 cases, 50.0%), exon 2l point mutations L858R (11 cases, 42.3%) and L861Q (1 case, 3.8%), and exon 18 point mutation G719X (1 case, 3.8%).

Conclusion Malignant pleural effusion cell blocks combined with immunocytochemical markers and molecular pathology are helpful for the diagnosis of advanced tumors, the identification of tumor properties and histological tumor origin, and the selection of individualized treatment for advanced lung cancer.

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Key words: pleural effusion; cell block; immunocytochemistry; epidermal growth factor receptor (EGFR)

Due to the location of the tumor or the occurrence of complications, it is difficult for a considerable number of patients with advanced tumors to obtain their tumor tissues through intervention [1]. Only a few cases with surgery can provide large specimens, and most cases are diagnosed based on small biopsy materials, such as fiberoptic bronchoscopy, percutaneous lung core biopsy, endobronchial ultrasound-guided transbronchial needle aspirate (EBUS-TBNA), and cytological examination. In comparison, cytological specimens are relatively easy to obtain, so the use of cytological specimens for corresponding detection is significant for the diagnosis of tumors and the guidance of clinical personalized treatment. In this study, the application value of a modified cell paraffin block technique in the individualized diagnosis and treatment of advanced non-small cell lung cancer (NSCLC) by combining tumor-related specific protein and gene detection was discussed.

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Materials and methods

Materials

The 65 samples from patients with malignant pleural effusion that were treated in the Department of Oncology of the Inner Mongolia People's Hospital (China) were collected from January 2016 to December 2016, including 31 males and 34 females, aged 29–88 years old, with an average age of 60.14 (± 16.94) years old.

Methods

The smear was prepared by conventional methods and stained with hematoxylin and eosin (H&E).

Cell block production

Specimen was refrigerated at 4 °C for two hours until visible precipitation formed. Supernatant was then removed, the precipitate was collected in a 50 mL tube, and centrifuged at 2000 r/min for five minutes, supernatant was removed, then was fixed in 15 mL of 10% neutral formaldehyde for two hours at room temperature, wrapped in filter paper, and submitted for dehydration, transparency, paraffin embedding, sectioning, and staining.

Immunocytochemical staining

The 3 μm-thick sections were prepared from the paraffin blocks, and the EnVision two-step staining method was carried out according to the instructions. Immunohistochemical antibodies were selected based on microscopic examination of morphology by smear and biopsy, evaluation of H&E sections, and corresponding clinical data for each case. For unknown origin of adenocarcinoma, a panel of antibodies with Napsin A, CDX-2, TTF-1, CK20, CK7, and Villin markers was selected. TTF-1, CA125, CDX-2, and GCDFP-15 can be used as organ-specific antibodies for lung adenocarcinoma, ovarian epithelial cancer, digestive tract adenocarcinoma, and breast cancer, respectively. For cases of mesothelial cells, Calretinin, WT-1, and D2-40 were added. The primary and secondary antibodies used were all purchased ready-to-use from Beijing Zhongshan Jinqiao Biotechnology Co., Ltd. (China), and the positive and negative controls used were purchased from Beijing Zhongshan Jinqiao Biotechnology Co., Ltd. (China).

Gene mutation detection

Cell paraffin blocks were made into eight sections of 8 μm-thick sections, and DNA was extracted by using the QIAamp DNA FFPE Tissue Kit according to the described steps. The concentration and quality of extracted DNA products were determined by the Thermo ND-2000 nucleic acid and protein quantitative analyzer, and the OD260/OD280 ratio of the DNA was required to be between 1.8 and 2.0. EGFR gene mutation was detected by the Xiamen AiDe AMRS kit, and the specific steps were performed on the ABIStep-one fluorescence quantitative PCR instrument according to the instructions. A positive control and a negative control were set for each test, and the reaction volume was set to 25 μL.

Statistical analysis

For the statistical analysis, the SPSS 17.0 software (IBM, USA) χ^2 test analysis was used, and $P < 0.05$ for the difference was considered statistically significant.

Results

Cell smear and cell block sections

Of the 65 samples, 50 were diagnosed with adenocarcinoma, 7 with lung squamous cell carcinoma, 6 with small cell lung cancer, and 2 with mesothelioma. Compared with the smear, the tumor cells in the cell block section were abundant in both quantity and quality, and some histologic characteristics were maintained. The squamous cells are nests, with intercellular bridges (Fig. 1). The morphology of cells in neuroendocrine cancer cells in the smear and section were similar: small cells, with sparse or absent cytoplasm, irregular hyperchromatic nuclei, easy mitosis, often single cell shedding, and different from the background lymphocytes.

Immunophenotype

The immunocytochemistry staining of cell block sections was accurate, and the granules were clear (Fig. 1 and Table 1).

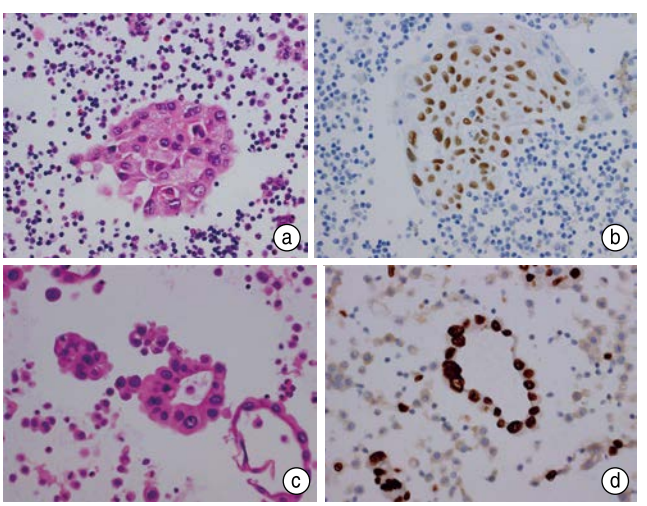

Fig. 1 (a) Squamous cell carcinoma nest in cell block with dense eosinophilic cytoplasm, individual keratinization and intercellular bridges; (b) Immunohistochemistry revels that the tumor cells are positive for p40; (c) Adenocarcinoma in cell block with glandular lumen formation, bubbling cytoplasm, and intracytoplasmic mucin; (d) Immunohistochemistry analysis reveals the tumor cells are positive for TTF-1 (all H&E staining, 400×)

Table 1 Immunocytochemical staining of 65 sample cell sections

Antibodies	Lung cancer			Breast cancer	Ovarian cancer	Colorectal cancer	Pleural mesothelioma
	Adenocarcinoma	Squamous cell carcinoma	Small cell carcinoma				
TTF-1	+	–	–	–	–	–	–
Napsin A	+	–	–	–	–	–	–
CK5/6	–	+	–	–	–	–	+
p40	–	+	–	–	–	–	–
CgA	–	–	+	–	–	–	–
CK20	+/–	–	–	–	–	+	–
CK7	+	–	–	+	+	–	–
villin	+	–	–	–	–	+	–
Calretinin	–	–	–	–	–	–	+
D2-40	–	–	–	–	–	–	+
WT-1	–	–	–	–	–	–	+
CDX-2	–	–	–	–	–	+	–
CA125	–	–	–	–	+	–	–
GCDFP-15	–	–	–	+	–	–	–

Gene mutation detection

In 47 NSCLC patients, EGFR mutation was detected in 26 cases (55.3%) by ARMS. Within these 26 samples, four mutation types were detected: exon 19 deletion (13 cases, 50.0%), exon 2l point mutations L858R (11 cases, 42.3%) and L861Q (1 case, 3.8%), and exon 18 point mutation G719X (1 case, 3.8%). All mutations were unipoint mutations and no multilocus mutations were found.

Discussion

According to the United States National Cancer Institute Surveillance, Epidemiology and End Results (SEER) database, 57% of lung cancer patients have already developed distant metastases [2] at the first diagnosis. In China, this proportion is higher, as most of the lung cancer patients have been diagnosed at an advanced stage [3]. Small specimens, including malignant serosal effusion cells, are an important way to clarify the diagnosis of the disease. However, due to its small sample size, the specimen needs to be properly handled to provide accurate pathological and molecular diagnosis.

Converting serosal effusion cells into cell block is an effective method to improve the cytological diagnosis [4]. Cell block sections showed less background, high quality of staining, and uniform thickness, and histologic features could be observed in some cases. For example, glandular lumen or papillary structures appeared in sections of adenocarcinoma; nests, intercellular bridges, or keratinocytes could be seen in squamous cell carcinoma; and spindle/oval shaped cells, scattered or molding, in small cell carcinoma (Fig. 1). The cell block can be used for diagnosis and classification of tumor, which is almost compatible to histological diagnosis. At present, the thrombin method, agarose method, and cell precipitation method are commonly used to prepare cell blocks, but some of these methods require the addition of other enzymes to the material, which may have an impact on subsequent analysis. Some production processes are more cumbersome. In this study, cell blocks were prepared by cold precipitation, without adding additional enzymes, and the protein components contained in the hydrops were connected into a network scaffold, which was conducive to the adhesion between cells. This allowed for a convenient processing method, with no interference to the subsequent detection. Additionally, the number of harvested cells was large and the production efficiency was high. 10% neutral formaldehyde is one of the most commonly used fixative solutions, but DNA can be easily affected by the fixation time. Furthermore, sample DNA soaked in formaldehyde for days will be fragmented and cannot be used to effectively detect mutations. In this study, the fixation time of the sample is no more than two hours, which is optimal for cell nuclear structure stability and antigen retention [5].

Immunocytochemistry plays an important role in defining the pathological classification, origin and differential diagnoses of given tumors [6–7]. For mesenchymal tumors and neurogenic tumors, the primary tumor has no organ specificity; therefore, the significance of finding the primary tumor is limited. However, for epithelial-derived tumors, especially adenocarcinoma, the search for primary lesions is of great significance. Cytokeratin (CK) is a component of the cytoskeleton protein family of filaments and a reliable marker of epithelial differentiation. Some antibodies have a certain degree of organ specificity, such as TTF-1 expression mainly in lung and thyroid epithelial cells [8], CDX-2 and CK20 in the gastrointestinal epithelium, and CA-125 as the main marker of epithelial ovarian cancer,

especially serous carcinoma. However, single antibody detection can be difficult to use to determine the tumor origin of many metastatic tumors, while the combined use of antibodies can narrow down the potential sites of the primary tumor to a greater degree [9]. CK7 and CK20 can identify the origin of most adenocarcinomas, but there are still deficiencies in practical applications. Villin, as a cytoskeleton protein, plays an important role in the formation of brush border microvilli of many cells. Villin is relatively conserved in the process of tumor formation, and its combined application with CK7 and CK20 can help to clarify the primary lesions of adenocarcinoma, though it has no obvious value for squamous cell carcinoma and small cell carcinoma. Malignant mesothelioma is a kind of bidirectional differentiated malignant tumor, which can express both keratin and vimentin. Mesothelioma cells and the lining cells in the serosal cavity, are variable in morphology and often confused with other tumor cells. For the differential diagnosis between pleural epithelioid malignant mesothelioma, lung adenocarcinoma, and squamous cell carcinoma, there is no entirely specific marker at present, so at least two mesothelium markers and two epithelial markers should be selected [10]. Depending on the sensitivity and specificity of the antibody, the best markers at present for mesothelioma are Calretinin, CK5 or CK5/6, WT-1 and D2-40.

The molecular subclassification of NSCLC based on tumor oncogenes and molecular targeted therapy for corresponding genes has become the standard treatment for advanced NSCLC due to its remarkable efficacy and excellent safety in patients. EGFR is a transmembrane receptor, which is related to cell proliferation, metastasis, and apoptosis, along with signal transduction pathways. There are four major EGFR mutations: exon 19 deletion, exon 21 point mutation, exon 18 point mutation, and exon 20 insertion [11].

The most common types of EGFR mutations are deletion of exon 19 and exon 21 L858R point mutations. Both of these lead to tyrosine kinase domain activation and are EGFR-TKI sensitive mutations. Exon 18 G719X, exon 20 S768I, and exon 21 L861Q mutations also are gain-of-function mutations, while the exon 20 T790M mutation is related to acquired drug resistance of EGFR-TKI. There are many other types of mutations [12]; however, the clinical significance of those mutations remains unclear. The positive rate of EGFR gene mutation in lung adenocarcinoma patients is about 10% in Caucasian population, and about 50% in east Asian population [13]. Multiple clinical studies have demonstrated that NSCLC patients with EGFR gene mutations benefit from EGFR-TKI treatment significantly [3, 14–15]. For cytological specimens of NSCLC patients, the concentration of DNA from tumor cells is low, and the mutation rate is not high. The amplification refractory mutation system (ARMS) is of high sensitivity and can detect mutant DNA with concentrations as low as 1%, and is also a closed detection system with near-zero cross contamination. In this study, out of 47 NSCLC patients, EGFR mutations were detected in 26 patients by ARMS, which is in line with the published data.

Cell block has advantages in immunohistochemistry analysis and is superior to cytology analysis alone. However, there are some disadvantages to this method. For example, the number of tumor cells in some cell blocks are limited. Heterogeneity of tumors in a given cell block could have test results that may not be completely representative. Immunocytochemical staining may be nonspecific if apoptosis is occurring. Additionally, tumor cells are sometimes mixed with benign mesenchymal cells and inflammatory cells including histiocytes, which requires careful observation and identification. Furthermore, in order to improve the success rate of ARMS detection, it is suggested that the quality of DNA extracted be monitored every time. The OD260/OD280 ratio of qualified samples should be between 1.7 and 2.0, and samples with impure or inadequate concentration should be extracted again to avoid false negative results, as an insufficient amount of DNA will affect the test results for some specimens.

Conflicts of interest

The authors indicate no potential conflicts of interest.

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