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Application of non-small cell lung cancer pleural effusion cell blocks in molecular pathological detection

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Abstract ***Objective:*** The tumor tissues used in molecular pathological detection were usually obtained by surgery, which would cause trauma and may not be suitable for the terminal cancer patients. This paper evaluated the value of the non-small cell lung cancer (NSCLC) pleural effusion cell blocks as tumor tissues replacement materials in the application of molecular pathological detection. ***Methods:*** Tumor cells were made into cell blocks through stratified centrifugal from 30 NSCLC patients with the pleural effusion. The immunohistochemistry, fluorescence in situ hybridization (FISH) and gene sequencing methods were employed in our experiments. ***Results***: The tumor cells of cell block section were rich and could keep part of histological structure. Immunohistochemistry staining could assist diagnosis and tumor parting. Epidermal growth factor receptor (EGFR) FISH-positive was found in 33.33% of the group, high polysomy in 6 cases, amplification in 4 cases. EGFR gene mutations were found in 8 cases of 30 samples, with an incidence of 26.67%, 6 cases were detected in the exon 19, and 2 cases were detected in the exon 21. ***Conclusion:*** The NSCLC pleural effusion cell blocks are useful for the diagnosis and determining the primary source of tumor, instructed targeted therapy.

Key words carcinoma; non-small cell lung (NSCLC); pleural effusion; cell block; immunohistochemistry; fluorescence in situ hybridization (FISH); mutation

Pleural effusion is one of the main complications in patients with advanced non small cell lung cancer (NSCLC) [1]. The conventional cytology smear staining was employed to judge the benign and malignant for the majority of pleural effusion. However, due to the lack of objective index, it is difficult to distinguish the hyperplastic mesothelial cells and adenocarcinoma cells, and even difficult to determine the sources and types of tumors. In some cases, the small number or deformation of cells with diagnostic significance, resulted from the limited samples with blood and jelly state, will lead to misdiagnose. So, the risk of choosing treatment by conventional cytology smear microscopy results is very high. It is also difficult to provide valuable information by common cytological smear due to the low positive rate and poor reproducibility. In the present work, the pleural effusion cell block technology, immunohistochemical staining, fluorescence in situ hybridization (FISH), and gene sequencing were employed to the molecular pathological diagnosis of terminal non small cell lung cancer in clinical works.

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Materials and methods

Overview

The 30 cases of NSCLC pleural effusion ranging June 2011–December 2012 in our hospital were taken as the samples in this work. Among these samples, there were 17 male patients and 13 female patients with average age of 55 (in the range of 23–86) years. In addition, there were 28 patients with pancreatic cancer and 2 patients with squamous cell carcinoma.

Cell block preparation methods

The cell block was prepared by three steps: (1) The pleural effusion specimens were kept still at room temperature for 10–20 min. 50 mL samples at the bottom of the container were extracted by 50 mL centrifuge tubes. After being centrifugal separation for 5 min with 2500

r/min, the sediment was preserved. (2) The 5–10 mL 10% neutral formaldehyde were added into the tube to mix with the sediment. After fixed processing at room temperature for about 1 h, the sediments were obtained by centrifugalism. The final sediments were got by repetitive operation of this step. If there were little amount of the sediments, 5–10 mL alcohol 75% would be added to solidification. (3) With absorbing cell block to the microscope lens wiping paper, the tissue processing was carried out in the automatic dehydration machine and the paraffin-embedded cell blocks were obtained. The paraffin-embedded cell blocks were cut into slices (with 3 μm and 8 μm in thickness), which were dried for 1 h at 60 ℃.

Immunohistochemical staining

The Envision two-step immunohistochemical staining technique was applied to the cell block slices with 3 μm in thickness. The antibodies include Ckpan, WT1, D2–40, CK7, TTF-1, CK5/6, and p63, which were monoclonal antibodies and obtained from Fuzhou Maixin Company (China). The phosphate-buffered saline solution was used as the negative control, and the positive slices were employed as the positive control.

FISH and gene sequencing

The pleural effusion samples of NSCLC were applied to the FISH detection. The K-protein and probe of GLP EGFR/CSP7 were respectively obtained from Roche (USA) and Beijing Jinpujia Company (China). The hybridization signals of interphase cells were investigated by OLYMPUS BX51-typed fluorescence microscope. The red and green signals respectively indicated the target gene epidermal growth factor receptor (EGFR) and control probe CSP7. The ratio, in the unit of 100 cells, was calculated from the numbers of red signals/numbers of green signals. The positive control was divided into two types: (1) EGFR gene amplification (ratio ≥ 2), with clusters of red signals; (2) high polysomy (ratio < 2), with more than 40% cells showing more than 4 red signals; (3) FISH postive control (ratio < 2) without amplification.

Gene sequencing

The gene sequencing was also applied to the detection samples of FISH. The cell block slices with 8 μm in thickness were put into the EP tubes. The digested tissues were extracted by Toyobo DNA Kits. The 10 μL polymerase chain reaction (PCR) reaction system was described as follows: (1) 1U TaqDNA polymerase wasadded into the 10 mL PCR tubes containing 1 mL genome. The predenaturation and denaturation temperatures were respectively 94 ℃(15 min) and 94 ℃ (20 s) with 35 periods. The annealing, amplification, and final amplification temperatures were 60 ℃ (30 s), 72 ℃ (7 min), and 72 ℃ (7 min), respectively. The PCR products were amplified to 20 μL system. The 1% agarose gel electrophoresis and ultraviolet were used to identification the amplified fragments. After purification, the gene sequencing of PCR products was carried out in the ABI3100-Avant-typed sequenator. The repetitions of CA in the 19th, 20th, 21th exons and the first intron of the EGFR gene were tested. The data were analyzed by Date collection software.

Results

Pathological features of NSCLC pleural effusion cells

In the conventional cytological smear, the cancer cells were single scattered or exhibit clustering three-dimensional structure, as shown in Fig. 1a. The cells were randomly arranged with surface tension and curved contour. The atypia of the cancer cells was obvious with high nucleo-cytoplasmic ratio, thick nuclear membrane, coarse nuclear chromatin, dark stained nucleus, and clear nucleolus. The cancer cells in the cell block slices are concentrated in distribution. They not only exhibited morphological characteristics of cancer cells, but also maintained histological structure, such as aciniform, mamilla, and beam and cable structures (Fig. 1b and 1c).

Molecular phenotypes of NSCLC pleural effusion cells

In the immunohistochemical staining of 30 cell block slices, the positioning was accurate and the particles were clear, as shown in Fig. 2. The cancer cells in cell block slices were Ckpan (+), WT1, and D2-40 (–), among which the adenocarcinoma was CK7 and TTF (+), and squamous cell carcinoma was CK5/6 and p63 (+).

FISH

FISH technology was employed to detect the EGFR genes of 30 NSCLC samples. 20 samples were FISH-negative (Fig. 3a) and 10 cases were FISH-positive (Fig. 3b). The positive rate was 33.33 %. In the FISH-positive samples, there were 6 samples were high polysomy and 4 samples were EGFR gene amplification.

Gene sequencing

Mutation was identified in 8 cases. The mutation rate of EGFR gene was 26.67% (8/30). 6 cases were detected in the exon 19. 2 cases were detected in the exon 21 (Fig. 3c).

Discussion

The pleural effusion is usually caused by cancer cells stimulation and tissue fluid reflux disorder, which is resulted by lymphatic obstruction and angiemphraxis due to thrombus. It is difficult to employ the conventional cyto-

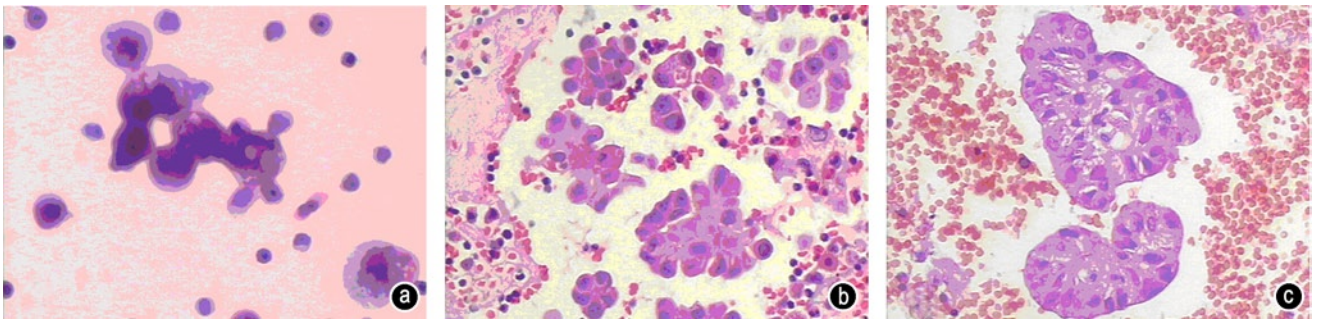

Fig. 1 The cytology of adenocarcinoma cells (HE staining × 200). (a) Cluster of adenocarcinoma cells in liquid-based cytology; (b) Papillary and (c) Adenoid structures in the tumor cells block

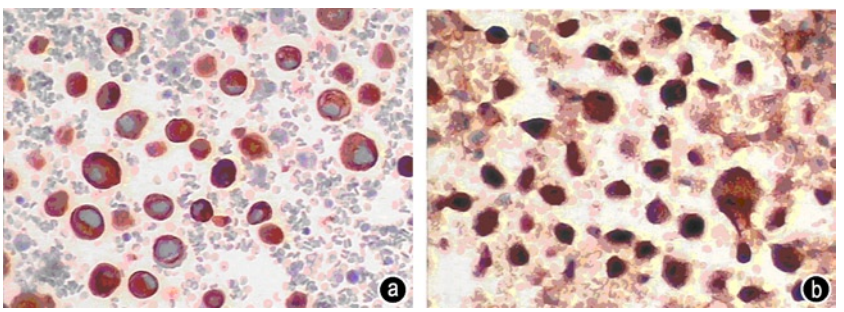

Fig. 2 (a) The granular cytoplasmic pattern of CK7 immunostaining (Envision × 200); (b) TTF-1 immunostaining with a nuclear pattern (Envision × 200)

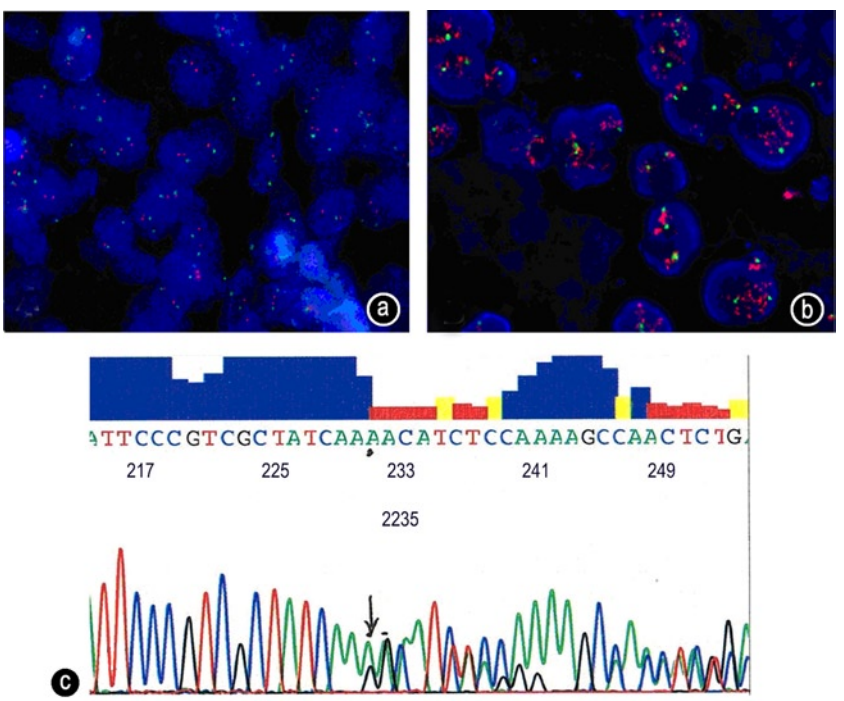

Fig. 3 (a) FISH analysis of EGFR gene without amplification; (b) TTF-1 immunostaining with a nuclear pattern (Envision × 200); (c) GFR gene amplification by FISH analysis. The direct sequencing result of exon 19 mutation in EGFR

logical smears to distinguish the hyperplastic mesothelial cells and adenocarcinoma cells, owing to that aggregation, cytoplasmic keratinization, and obscure intracellular mucin of the cancer cells. In addition, in the conventional cytological smears, the decentrality of cancer cells leads to low positive rate of immunohistochemistry staining and the poor repeatability. In the cell blocks made from the NSCLC pleural effusion cell, there are a large amount of cancer cell with clear structure and alignment. This is benefit to the routine pathological diagnosis [2–4].

The tumor cells, which intensively distribute in the cell block slices, maintain the antigenic specificity of the primary tumor. Thus, the immunohistochemical staining procedure can be accurately positioned and the positive

rate is high. The cell block slice is effective to auxiliarily diagnose the tumors as well as their type [5–13], provide a useful tool for the identification of tumors. Furthermore, it is also convenient to employ the cell block slices as screening of targeted therapy drugs, due to that the cell block can be long storaged and repetitive used.

In recent years, individualized treatment of NSCLC has been one of the focuses in the field of cancer research [14]. It has been reported that the targeted therapy can decrease the tumor size, improve the life quality of the patients, and prolong the survival period. The targeted treatment has been one of the most effective therapeutic methods for the advanced NSCLC with several times of failure of chemotherapy. However, the international multicenter and randomized clinical trial results show that the overall efficiencies of targeted drug are only 10–30 %. These results indicate that the targeted drugs are applied only to a part of patients with lung cancer. In the clinical treatment, the patient medication should be determined according to the individual difference, and consequently, the gene detection of tumor components is necessary. There are two kinds of therapeutic methods taking EGFR as the molecular targets: (1) EGFR-TKIs, including Geftinit, Erlotinib, and so on; (2) Cetuximab with the trade name of Erbitux.

FISH is an effective method to detect the increased EGFR gene copy number [15–23]. The amplification and expression levels of EGFR can be used as a routine examination to screen the drug of cetuximab. Cetuximab plays a therapeutic role through directly binding to EGFR protein so as to block the signal pathway. The random FLEX clinical trials at phases II and III shows that the Cetuximab in combination with the chemotherapy can obviously prolong the survival of high EGFR expression patients with NSCLC. Usually, the tumor tissues used in molecular pathological detection were obtained by surgery, which would cause trauma and not suitable for the terminal cancer patients.

In this work, the cell blocks were made from the pleural effusion. The FISH results exhibit clear fluorescence signal, obvious red-green contrast, clear contours of DAPI counterstained nucleus. So, it is convenient to analyze the fluorescence microscopy and the positive rate is achieved to 33.33 % in the selected 30 samples in this work.

However, it is still controversial on the values of EGFR amplification in the prediction of EGFR-TKIs. In the treatment based on EGFR-TKIs, EGFR amplification is not the routinely commended to be detected. For the tumor tissues, the gene sequencing is one of the most valuable indicatior to screen the TKIs object and predict the treatment response [24–31]. The effective rate of EGFR-TKIs on EGFR mutant is above 80%. But there is no effect of EGFR-TKIs on the wild-type tumors. In the 30 NSCLC pleural effusion cell blocks, the percentage of gene mutation is 26.67 %, indicating that the DNA extracted from the pleural effusion, especially from the patients with advanced lung cancer, can also be an effective approach to detect the EGFR. The activation and mutation of EGFR usually occur in the 18–21th exon (E18-21), in which the deletion of E19 (746–750) and mutation of E21 (L858R) account for about 90% of all mutations. The different mutations exhibit different biological functions. The E19 deletion and E21 mutation indicate obvious response to the EGFR-TKIs. E20 point mutation (T790M) is one of the important mechanisms for the formation of EGFR-TKIs secondary resistance. Gene sequencing has a high requirement to the content of tumor cells in the specimens. Especially in the case of small biopsy or cytology specimens, the content of tumor cells is required to be higher than 50 %. If the content is lower than 25%, the probability of false negativity will dramatically increase [32].

In conclusion, the pleural effusion cell block is a noninvasive specimen collection method for the molecular pathology detection. The cell block, with long-term storage and measurement repeatability, is an effective approach for histological classification and gene detection. It is demonstrated that the cell blocks provide a useful tool for molecular pathological diagnosis for the lung carcinoma at the advanced stage and targeted cancer therapy.

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