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Reliability of a Tissue Microarray in Detecting Thyroid Transcription Factor-1 Protein in Lung Carcinomas

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OBJECTIVE To compare the expression of the thyroid transcription factor-1 (TTF-1) in human normal adult type II alveolar epithelial cells, embryonic pneumocytes and cancer cells of lung carcinoma and metastatic lymph nodes using a tissue microarray (TMA) along with paired conventional full sections, and to investigate the reliability of tissue microarrays in detecting protein expression in lung carcinoma.

METHODS A lung carcinoma TMA including 765 cores was constructed. TTF-1 protein expression in both TMA and paired conventional full sections were detected by the immunohistochemical SP method using a monoclonal antibody to TTF-1. A PU (Positive Unit) of TTF-1 protein was assessed quantitatively by the Leica Q500MC image analysis system with results from the paired conventional full sections as controls.

RESULTS There was no significance between TMA and paired conventional full sections in TTF-1 expression in different nuclei of the lung tissue.

CONCLUSION TTF-1 protein expression in lung carcinoma detected by TMA was highly concordant with that of paired full sections. TMA is a reliable method in detecting protein expression.

KEYWORDS: lung carcinoma, tissue microarray, thyroid transcription factor-1(TTF-1), immunohistochemistry, reliability.

INTRODUCTION

The original method of in situ detection of protein expression in paraffin-embedded tumour tissues involves immunohistochemical staining on a conventional full section. This allows the detection of only one specimen on a glass slide each time. Errors may occur because of different experimental conditions between different groups and batches and it consumes considerable time and reagents.

A tissue microarray, also called a tissue chip[1], is an ordered array of hundreds to thousands of tissue cores in a single paraffin block. Consecutive sections cut from the block provide the starting material for the simultaneous and unbiased in situ detection of a gene or protein targets in a very large number of tissue samples for clinical or basic research. It is highly efficient, allowing thousands of genes to be monitored simultaneously for expression level and comparisons to be made among many different tissues on a single glass slide. However, because of the small size of the biopsies used in the tissue microarrays, 0.6 mm in diameter, and due to the heterogeneity of most tumors, it raises the question as to whether very small tissue samples may always reflect the biological properties of the entire tumor. To elucidate the reliability

of tissue microarrays in detecting protein expression in lung carcinoma, we investigated the expression of the thyroid transcription factor-1 (TTF-1) in human lung carcinomas using tissue microarrays (TMAs) and paired conventional full sections (CFS) by immunohistochemistry and a quantitative image analysis technique[2-4].

MATERIALS AND METHODS

Materials

Paraffin-embedded specimens were from the Pathology Department of the Nanfang Hospital. The specimens were classified into two groups, i.e. a conventional full-section group and a tissue microarray group. Both groups were comprised of 20 normal adult lung tissues, 15 embryonic lung tissues and 100 lung carcinomas. We determined histological typing and grading (well, moderately, and poorly differentiated) of the lung carcinomas according to the WHO/IASLC classification[5], including 35 adenocarcinomas (21 well-differentiated and 14 moderately to poorly differentiated), 40 squamous cell carcinomas (23 well-differentiated and 17 moderately to poorly differentiated), 14 small cell lung carcinomas and 11 large cell lung carcinomas). There were 55 cases of lung carcinomas with lymph node metastases, 20 metastases from adenocarcinomas, 24 from squamous cell carcinomas, 6 from small cell lung carcinomas and 5 from large-cell lung carcinomas. Fifty-nine lung carcinomas were a peripheral type and 41 were a central type. Sixty-two lung carcinomas were in TNM Stage I and 38 were in Stages II~IV. All paraffin-embedded tissue blocks were used as donor blocks.

Tissue microarray construction

For tissue microarray construction, a hematoxylin and eosin (H&E)-stained section was made from each donor block to define representative tumor regions. Tissue cylinders with a diameter of 0.6 mm were then punched from representative areas of each donor tissue block and brought into a recipient paraffin block using a custom-made precision instrument (Beecher Instruments, MTA-1). Four tissue cores were randomly selected from the defined regions of each donor block. Location of the individual tissue core in the two-dimensional arrays in the recipient paraffin block was recorded. A 25×30 matrix was constructed with 15 tissue cores in the last row as an indication of the location. Samples for all specimens were distributed in one regular-sized paraffin block according to the following order: 20 normal lung tissues (positive controls), 15 embryonic lung tissues, 35 primary lung adenocarcinomas, 20 lymph node metastases of lung adenocarcinomas, 40 primary squamous cell lung carcinomas, 24 lymph node metastases of squamous cell lung carcinomas, 14 primary small cell lung carcinomas, 6 lymph node metastases of small cell lung carcinomas, 11 primary large-cell lung carcinomas and 5 lymph node metastases of large cell lung carcinomas. Primary sites of different lung carcinomas were distributed from metastatic to non-metastases ones.

The specific processes were as follows: ①H&E stained sections were observed carefully under a microscope to localise the representative regions and marks were made both on the glass slide and corresponding tissue blocks using a DAKO marker pen. ②Paraffin wax (melting point: 60℃) was from the Shunhe Wax Factory of Maoming City, Guangdong Province, China. A blank paraffin block with a matrix of 35 mm×25 mm×5 mm was made as a recipient tissue block. ③Holes were first made with a manual arrayer that used core needles of 0.6 mm in diameter for punching a blank paraffin block with a space of 0.66 mm between two cores and a depth of 3 mm. The needle was used to retrieve a cylindrical sample from a selected region in the donor block and extrude the sample core directly into a recipient block with defined array coordinates. ④The sampling cycle was repeated to punch different regions in the donor blocks. ⑤The ready-made tissue microarray was warmed to 37℃ for 30 min, pressed using a clean glass slide to make the surface even, and then cooled to room temperature. ⑥The resulting tissue microarray block was cooled to -20℃ for 30 min and then 3 μm sections of the block were cut with a microtome. The sectioned paraffins were then mounted on poly-L-lysine-coated slides (Sigma Company) and heated at 60℃ for 3 h to make the paraffin sections and slides stick tightly followed by storage at 4℃ until used. The first section of the tissue microarray block was stained with H&E to identify the precision of the construction processes.

Immunohistochemistry

Slides of the tissue microarray and conventional full sections were stained with antibodies to TTF-1 (Clone: 8G7G3/1, working solution, Maxin Co., Ltd., Fuzhou, China) based on the standard streptavidin-biotin complex method.

Immunohistochemical staining was carried out on 3 μm thick sections cut from the constructed tissue microarray. Sections were deparaffinized with xylene, rehydrated with a graded series of ethanol solutions and rinsed in PBS (pH 7.4). Deparaffinized sections were first placed in plastic jars filled with citrate buffer (pH 6.0) and heated for 10 min at 120℃ in an autoclave. After autoclave pretreatment, the sections

were allowed to cool to room temperature and then were exposed to 3% hydrogen peroxide in water to inactivate endogenous peroxidase.

Indirect immunoperoxidase staining was used according to standard protocols. The sections were incubated for 30 min at 37℃ with normal nonimmune serum and then incubated at 4℃ with anti-TTF-1 antibody for 12 h. The sections were washed in PBS three times and treated with biotin-conjugated second antibody before adding streptavidin-peroxidase. The peroxidase reaction was performed using 3,3'–diaminobenzidine tetrahydrochloride (3,3'–diaminobenzidine).

Normal lung tissues were used as an internal positive control. PBS instead of the anti-TTF-1 antibody and muscle tissues were used as negative controls.

Image analysis of the TTF-1 protein

The amount of TTF-1 protein was measured by the Leica Q500 MC image analysis system. On the premise that there were no false positives or false negatives, those with dark-brown particles in the nuclei were regarded as positive protein expression. Detected with a 40× object lens, 20 fields were randomly selected within each specimen on the conventional full sections and within 4 randomly chosen representative tissue cores of a specimen on the tissue microarray sections. For the normal lung tissues, 10 random positive-type II pneumocytes were measured in each visual field and for the embryonic lung tissue, 10 random pneumocytes were measured in each visual field. For lung carcinomas and metastatic lymph nodes, 10 random-positive lung neoplastic cells were measured. Two hundred positive cells were measured in each specimen. An interactive method was used to measure the gray level (G_α) of each positive cell and background gray level (G_β) was measured in the same field. Based on the formula below, a positive unit (PU) for each positive cell was calculated. The average value of the PU of 200 positive cells was regarded as the positive unit of the specimen[2-4].

$$PU = \frac{|G_\alpha - G_\beta|}{256} \times 100$$

Statistical analysis

The SPSS 10.0 software package was used for statistical analysis. TTF-1 protein expression among the six groups was compared using ANOVA ($P<0.05$). Comparisons between metastatic and non-metastatic groups of lung carcinomas were analyzed for statistical significance ($P<0.05$) by the student t test.

RESULTS

An overviewing of the staining

The paraffin-embedded tissue microarray block, a scanned picture of the slide and an H&E-stained microarray section are shown in Figs.1~3.

Table 1. Designed and valid disks in the paraffin tissue microarray.

Tissues	Cases	Valid/Designed	Valid Rate (%)
Muscular tissues	5	5/5	100
Normal adult lung tissues	20	73/80	93
Embryonic lung tissues	15	60/60	100
Lung carcinoma			
Adenocarcinoma			
Primary site	35	138/140	98
Lymph node metastases	20	76/80	97
Squamous cell lung carcinoma			
Primary site	40	159/160	98
Lymph node metastases	24	93/96	96
Small cell lung carcinoma			
Primary site	14	56/56	100
Lymph node metastases	6	23/24	95
Large cell lung carcinoma			
Primary site	11	42/44	97
Lymph node metastases	5	18/20	95
Total		743/765	97

H&E staining results of the tissue microarray

Results of H&E staining sections of the tissue microarray with 765 disks were recorded as follows. Nine disks were not representative of the desired regions (3 metastases of lung adenocarcinoma, 3 metastases of squamous cell lung carcinma, 1 metastasis of a small cell lung carcinoma, and 2 metastases of large cell lung carcinoma). Three disks were incomplete (2 normal lung tissues and 1 primary site of lung adnocarcinoma). Three disks were missing (1 normal lung tissue, 1 metastases of lung adenocarcinoma and 1 primary site of large cell lung carcinoma). The valid rate was 98% (750/765). Disks were arrayed in order with moderate thickness, distinctive staining and apparent demarcation. After immunohistochemistry, 6 more disks were missing (4 normal lung tis-

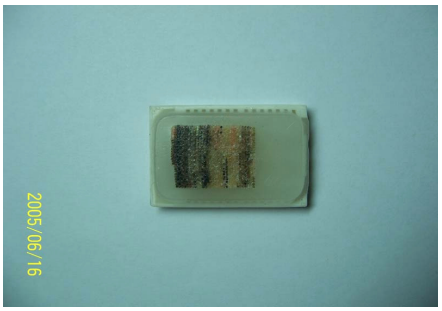

Fig.1 Paraffin-embedd block of the tissue microarray (765 disks, 0.6 mm in diameter)

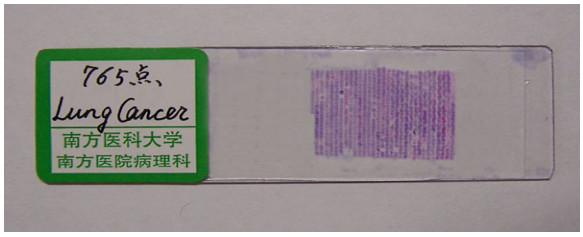

Fig.2 Glass slide of the tissue microarray (765 disks, 0.6 mm in diameter)

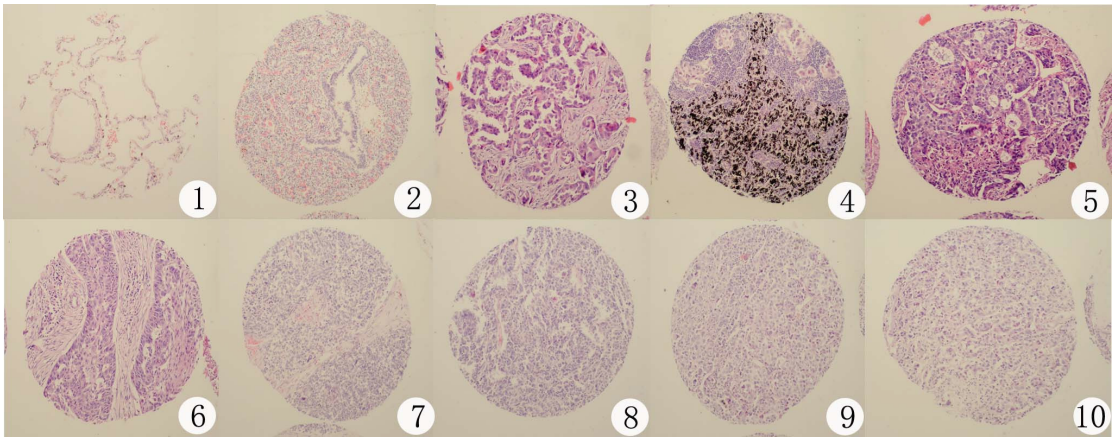

Fig.3 H&E stained sections of the tissue microarray (×100) ①Normal adult lung; ②Embryonic lung; ③Primary lung adenocarcinoma; ④Lymph node metastases of the lung adenocarcinoma; ⑤Primary squamous cell lung carcinoma; ⑥Lymph node metastases of squamous cell lung carcinoma; ⑦Primary small cell lung carcinoma; ⑧Lymph node metastases of small cell lung carcinoma;⑨Primary large cell lung carcinoma;⑩Lymph node metastases of large cell lung carcinoma.

Table 2. TTF-1 protein expression in the nuclei of type II pneumocytes of normal lung, embryonic pneumocyte and lung cancer cells in conventional full sections(CFS) and tissue microarray(TMA).

Groups	Cases	TMA	CFS	*P*
Normal adult lung (a)	20	$35.11\pm2.83^{b,c1,c2,c3,c4}$	$36.01\pm1.52^{b,c1,c2,c3,c4}$	>0.05
Embryonic lung tissues (b)	15	$21.62\pm1.87^{b,c1,c2,c3,c4}$	$21.16\pm0.92^{a,c1,c2,c3,c4}$	>0.05
Lung carcinoma				
Adenocarcinoma (c1)	35	$13.62\pm1.62^{a,b,c2,c4}$	$12.22\pm1.63^{a,b,c2,c4}$	>0.05
Squamous cell carcinoma (c2)	40	$4.22\pm1.11^{a,b,c}$	$4.74\pm0.74^{a,b,c4}$	>0.05
Small cell carcinoma (c3)	14	$14.07\pm1.96^{a,b,c2,c4}$	$13.17\pm0.93^{a,b,c2,c4}$	>0.05
Large cell carcinoma (c4)	11	$3.11\pm0.81^{a,b}$	$3.51\pm0.27^{a,b}$	>0.05

Superscript (a, b, c1, c2, c3, and c4): $P<0.001$ *vs.* Group a, b, c1, c2, c3, and c4 respectively.

Table 3. TTF-1 protein expression in primary and metastatic sites of lung carcimomas in conventional full sections (CFS) and tissue microarray (TMA).

Items	Primary site				Lymph node metastases			
	No.	TMA	CFS	*P*	No.	TMA	CFS	*P*
c1	35	13.62±1.62	12.22±1.63	>0.05	20	16.34±1.24^{c2,c3,c4}	16.49±0.86^{c2,c3,c4}	>0.05
c2	40	4.22±1.11	4.74±0.74	>0.05	24	8.97±1.96^{c1,c3}	8.21±1.86^{c1,c3}	>0.05
c3	14	14.07±1.96	13.17±0.93	>0.05	6	13.79±1.05^{c1,c2}	13.22±0.86^{c1,c2}	>0.05
c4	11	3.11±0.81	3.51±0.27	>0.05	5	5.70±2.73^{c1}	6.47±3.69^{c1}	>0.05
Total	100	8.77±5.11	8.40±4.21	>0.05	55	11.88±4.23	11.61±4.42	>0.05

Superscript (c1, c2, c3, and c4): *P*<0.001 *vs.* group c1, c2, c3, and c4 respectively.

Table 4. Comparisons between TTF-1 PU and clinicopathological characteristics of lung carcinomas.

Items	No.	TMA	CFS	*P*
Gender				
Male	61	13.57±1.93	13.52±1.77	>0.05
Female	39	14.21±1.11	13.85±1.18	>0.05
General types				
Peripheral	59	13.62±1.13	13.64±1.03	>0.05
Central	41	13.88±1.44	13.37±1.42	>0.05
Differentiation adenocarcinoma				
Well differentiated	21	14.75±1.05	14.68±1.17	>0.05
Moderately to poorly differentiated	14	15.11±1.30	15.08±1.57	>0.05
Squamous cell carcinoma				
Well differentiated	23	5.98±2.30	6.09±2.24	>0.05
Moderately to poorly differentiated	17	4.97±1.95	4.81±2.35	>0.05
Lymph node metastases				
With	55	13.84±2.29*	14.17±2.08*	>0.05
Without	45	8.68±2.41	9.03±1.98	>0.05
TNM Stage				
Stage I	62	10.60±1.62*	10.85±1.26*	>0.05
Stages II~IV	38	14.50±2.36	15.09±2.07	>0.05

*P<0.001

sues, 1 primary site of lung adenocarcinoma and 1 primary site of squamous cell lung carcinoma) and 1 more was incomplete (large cell lung carcinoma). As is shown in Table 1, the overall valid rate was 97% (743/765).

TTF-1 protein expression in the conventional full sections and tissue microarray

As is shown in Table 2, there was no significance difference between the conventional full sections and tissue microarray in TTF-1 protein expression (P>0.05).

TTF-1 protein expression in primary and metastatic sites of lung carcinomas in conventional full sections and tissue microarray

Table 3 shows that TTF-1 protein expression is similar in metastatic sites of lung adenocarcinoma (c1), squamous cell lung carcinoma (c2), small cell lung carcinoma (c3) and large cell lung carcinoma (c4) comparing conventional full sections and the tissue microarray (P>0.05).

Relationships between TTF-1 protein expression and pathological characteristics of lung cancinomas in conventional full sections (CFS) and tissue microarray (TMA)

There was no significant difference between conventional full sections (CFS) and the tissue microarray (TMA) in TTF-1 protein expression (P>0.05, Table 4).

DISCUSSION

The conventional method of in situ detection of proteins or genes is to embed one tissue into a paraffin block, a procedure, which is laborious and time consuming. This method lowers the efficiency and speed of investigating a large number of tissue samples. A tissue microarray, also called a tissue chip, is an ordered array of hundreds to thousands of tissue cores in a single paraffin block. Consecutive sections cut from the block provide the starting material for the simultaneous and unbiased in situ detection of gene or protein targets in a very large number of tissue samples for clinical or basic research. This not only raises the experimental efficiency but also avoids the errors caused by grouping and batching. The accuracy of testing different specimens on a tissue microarray is raised. Results obtained by using tissue microarrays are more objective and comparative than by conventional full sections.

Schraml et al.[6] tested the amplification of ERBB2, CMYC and CCND1 by FISH in six different kinds of tumor tissues on a tissue microarray with 397 disks. They compared the results obtained from the tissue microarray and conventional full sections and found it to be a rapid way of detecting tumor markers with higher efficiency and accuracy. Fernebro et al.[7] obtained the same results in studies of P53 protein expression in 20 rectal carcinomas on both tissue microarray and conventional full sections. Richter et al.[8] constructed 5 tissue microarrays with 2,317 disks and finished the detection of CCNE gene amplification and protein expression in two weeks.

Studies[9-13] have shown that the tissue microarray technique to be a reliable way of detecting gene or protein expressions. We randomly selected 20 fields in the randomly chosen tissue disks from the representative regions of each specimen on the tissue microarray, with the finding that the TTF-1 PU of different nuclei was in concordance with those obtained from conventional full sections in our previous research[14](P>0.05). This shows that the tissue microarray with 765 disks in it can represent the characteristics of each separate tissue block. We used a positive unit (PU) in our research to evaluate the intensities of the TTF-1 protein in the nuclei after immunohistochemical staining. For the representative 4 disks selected from each specimen, all except those missing during the experiment were included in quantitative measurements. The consistency between conventional full sections and tissue microarray can be revealed using immunohistochemistry and quantitative analysis. Disks chosen from each specimen can represent the characteristics of the tumor itself. The validity rate of the tissue microarray with 4 disks selected from each paraffin block in our study was 97%. This shows that the tissue microarray can provide enough information for quantitative analysis.

We investigated the H&E stained sections of the tissue microarray and found that 15 disks were excluded from the experiment because they were incomplete, missing or unrepresentative of the desired regions. Seven more disks were missing or incomplete during immunohistochemistry. Four disks, randomly selected from the representative regions of each paraffin block, provided enough information for statistical analysis in our study. So we assumed that more disks were obtained from the paraffin block in the study so that this could make up for the loss of information caused by various factors such as construction of the tissue microarray or experimentation. Disks on the tissue microarray made from homemade paraffin were arrayed in order. So it is advisable to use a homemade paraffin block[15] to construct the tissue microarray. We found that normal lung tissues had the highest missing rate of 30% (7/22) compared to disks from other tissues. Sections of 3 μm of the tissue microarray block with a disk diameter of 0.6 mm were cut. The loose texture, isolate and discontinuous locations of the normal lung tissues make it more likely to get missed or incomplete during the procedure. So, more representative biopsies should be drawn from these donor blocks having a loose texture when constructing a tissue microarray.

We found an interesting phenomenon during the immunohistochemical staining. After the autoclave pretreatment, the peripheral tissues of conventional full sections floated, whereas isolated tissue disks on the tissue microarray were firmly stuck on the glass slide except for those incomplete or missing. This may occur because the margins of the disks were round and regular with an area of $\pi \times 0.32$ mm^2. It is easier for smaller disks to stick on the glass slide than larger and irregular full sections. So, using the tissue microarray technique could solve the problem of the floating tissue sections during antigen pre-treatment. We noticed that margins of the tissue disks were dry during the experiment. To deal with this problem, we

covered the disks with lens paper, which was filled with anti-TTF-1 antibody so that disks were most and totally exposed to the antibody.

We spent 4 days on tissue microarray construction, sectioning and immunohistochemical staining. Reagents used for the immunohistochemistry on the tissue microarray were 1% of those used in conventional full sections. TTF-1 protein expression detected on the tissue microarray was in high concordance with that detected on conventional full sections. We confirmed that results obtained from the tissue microarray can represent the results available from corresponding conventional full sections. The tissue microarray technique is a reliable way of conducting molecular biological research, and is a method which deserves wide application.

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