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Original Article

Use of automated image analysis in evaluation of Mesothelioma Tissue Microarray (TMA) from National Mesothelioma Virtual Bank

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ABSTRACT

The National Mesothelioma Virtual Bank (NMVB) was established to provide annotated biospecimens to the mesothelioma research community. The resource provides tissue microarrays (TMA) to evaluate the biomarkers along with a variety of other resected mesothelioma specimens. In this manuscript, we describe the immunohistochemical evaluation of the mesothelioma TMA with three key antibodies that are used in making the diagnosis of mesothelioma, and compared the immunohistochemical assessment between manual scoring and image analysis.

The TMA was assessed for the immunohistochemical expression of calretinin ($N = 39$), cytokeratin (CK) 5/6 ($N = 33$), and D2-40 ($N = 37$). Immunohistochemistry was evaluated by semi-quantitative (manual) scoring using light microscope (MS) and by automated image analysis (AS).

Calretinin staining was seen in both cytoplasmic and nuclear locations. CK5/6 stain was localized to the cytoplasm. D2-40 stain showed only membranous expression in our cases.

- Based on the pathologist's scores, calretinin was positive in 31 of the 39 cases (80%), CK 5/6 in 15 of the 33 cases (46%) and D2-40 in 18 of the 37 cases (49%).
- The percent-positive agreement between manual scores and image analysis was 90% (35/39), 94% (31/33), and 95% (35/37) for calretinin, CK 5/6, and D2-40, respectively. There was a substantial agreement between manual and automated scores for calretinin (kappa = 0.614) and an almost perfect agreement for CK5/6 (kappa = 0.879) and D2-40 (kappa = 0.892).

Our study confirms that the immunohistochemical staining pattern of mesotheliomas in the NMVB UPMC TMA is similar to other studies. Our findings also show that automated image analysis provides similar results to manual scoring by pathologists, and provides a reproducible, objective, and accurate platform for immunohistochemical assessment of biomarker expression.

Background

The National Mesothelioma Virtual Bank (NMVB) has proven to be a valuable tissue and data resource for the mesothelioma research community [1]. The resource has provided investigators with hundreds of specimens, including fresh frozen, paraffin, and tissue microarrays (TMAs). We perform regular quality assurance on specimens that we share with the investigators. In this study, we performed an immunohistochemical evaluation of the University Pittsburgh Medical Center (UPMC) TMA with three key antibodies that are commonly used in making the diagnosis of Mesothelioma [2]. We also compared the agreement of the immunohistochemical scores between manual scoring and image analysis for each of the three antibodies.

Materials and methods

NMVB patient

NMVB UPMC TMA was constructed to facilitate identification of biomarkers differentially expressed in malignant mesothelioma lesions. Samples from 40 cases have been incorporated in this TMA. Thirty six cases have tissue cores from the malignant mesothelioma, including three cases with tissue cores from metastatic lesion only and one case with tissue cores from both the primary and metastatic lesions. Four cases have a benign mesothelioma

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Table 1
Presents an overview of mesothelioma cases incorporated in the developed of TMA as per their anatomic site and histological type.

Anatomic site	Number of cases
Pleural	25
Peritoneum	13
Other (extra-pleural/peritoneal)	2
Histologic type	**Number of cases**
Biphasic	10
Epithelial/epithelioid	20
Sarcomatoid	3
Multicystic	1
Papillary	2
Benign fibrous	2
Fibrocystic	1
Desmoplastic	1

diagnosis. Of the patients with available clinical annotation, 97% are male and 3% are female, and racial breakdown is as follows: 57% white, 3% black, and 39% unknown. The pathologic site and the histologic type for annotated cases are shown in Table 1.

NMVB TMA construction

Archival tissue from 40 patients with mesothelioma from the Pathology department at the University of Pittsburgh Medical Center (UPMC) were used to construct a TMA with 0.6 mm tissue cores. The TMA was constructed using a manual arrayer (Beecher Instruments Inc., San Prairie, WI) [3].

Immunohistochemical staining

Immunohistochemical staining was performed using the DAKO Autostainer Plus instrument on 5 μm sections of the TMA using polyclonal rabbit antibody for calretinin (Biocare Medical, USA) at 1:200 dilution, mouse monoclonal antibody for D2-40 (clone D2-40, Biocare Medical, USA) at 1:200 dilution, and mouse monoclonal antibody for CK 5/6 (clone CK 5/6.007, Biocare Medical, USA) at 1:100 dilution [4,5]. Antigen retrieval was performed using Reveal (Biocare Medical, USA) for calretinin and D2-40, and using Borg (Biocare Medical, USA) for CK 5/6. Background Sniper (Biocare Medical, USA) was used as the blocking reagent for reducing non-specific antibody binding, MACH 2 Rabbit HRP-polymer (Biocare Medical, USA) was used as the secondary antibody, diaminobenzidine (DAB+) was used as the chromogen, and Harris Hematoxylin was used as the counterstain for calretinin, D2-40, and CK 5/6.

Immunohistochemical assessment

Immunohistochemical expression of calretinin, CK 5/6, and D2-40 was assessed both by semi-quantitative (manual) scoring using light microscope (MS) and by automated image analysis (AS).

For the semi-quantitative assessment, one experienced pathologist (MS) scored the immunohistochemical expressions using a method similar to the one described by Hinterberger et al. [6]. Briefly, both staining intensity and proportion of positive staining tumor cells were assessed. Staining intensity of tumor cells was classified as negative, weakly positive, moderately positive, and strongly positive. Weakly positive staining was defined as any faint staining intensity absolutely weaker than control tissue. Positive staining intensity was defined as staining comparable to control tissue. Strongly positive staining intensity was defined as staining absolutely stronger than control tissue. The percentage of tumor cells showing positivity was classified as negative if less than 10% of tumor cells were positive; as focal if between 10% and 30% of tumor cells stained, and as diffuse if more than 30% were positive. A final composite score combining the intensity and percentage-positive cells was given as follows: tumor spots with no staining or less than 10% staining were considered as negative (0); spots with weakly positive staining and focal distribution as moderately positive (1+), and spots with strong positivity and diffuse distribution as moderately positive (2+).

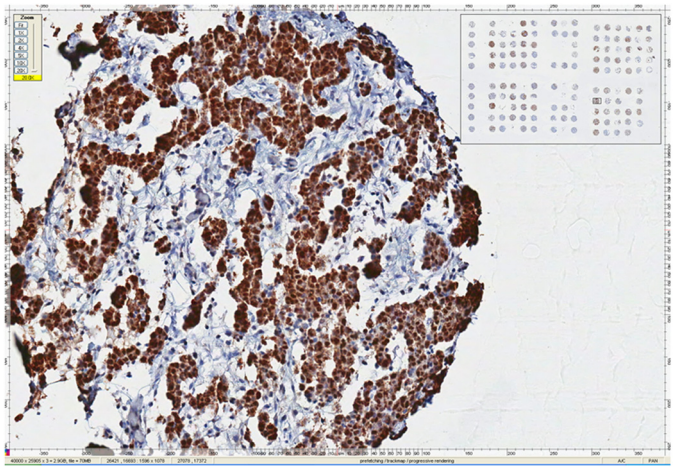

Fig. 1. Calretinin immunohistochemical staining of a mesothelioma histospot showing nuclear and cytoplasmic expression. A thumbnail image of the TMA is also seen in the background.

For image analysis, the TMA slide was digitized using Aperio ScanScope XTslide scanner (Aperio Technologies, Vista, CA) at 20× magnification [7]. The tumor areas in each histospot on the TMA were manually annotated using Aperio's annotation software (ImageScope v11.1.2.760, Aperio Technologies). Calretinin and CK5/6 were assessed using Aperio's Positive Pixel Count algorithm v9, and D2-40 was assessed using Aperio's Membrane algorithm v9.

A case was considered positive if at least one core was positive. Cores that lacked tumor or were not interpretable were excluded from the analysis. For the final analysis, there were 39 cases for calretinin, 33 cases for CK 5/6, and 37 cases for D2-40 (Figs. 1–3).

Statistical analysis

The agreement between pathologist's semi-quantitative scores and automated scores for each of the three immunohistochemical stains, calretinin, CK5/6, and D2-40, was assessed separately and a kappa statistic calculated to correct for chance agreement. To assess the agreement, for each immunohistochemical stain, we categorized the pathologist's scores into positive (1+ and 2+ composite score) or negative (score of 0). The automated scores for each stain were also categorized into positive or negative to enable comparison with the pathologist's scores. For the membrane algorithm (D2-40), similar to the categorization of the pathologist's score, the score generated by the algorithm was dichotomized into negative (when less than 10% of the cells exhibited staining) and positive (>10% cells exhibiting weak, moderate, or strong staining). For the positive pixel algorithm (calretinin and CK5/6), weak positive pixels were considered negative, and moderate and strongly positive pixels were considered positive. The kappa values were interpreted as follows: value of 0.81–1 as almost perfect agreement, 0.61–0.8 as substantial agreement, 0.41–0.60 as moderate agreement, 0.21–0.40 as fair agreement, 0.00–0.20 slight agreement, <0.00 as poor agreement [8].

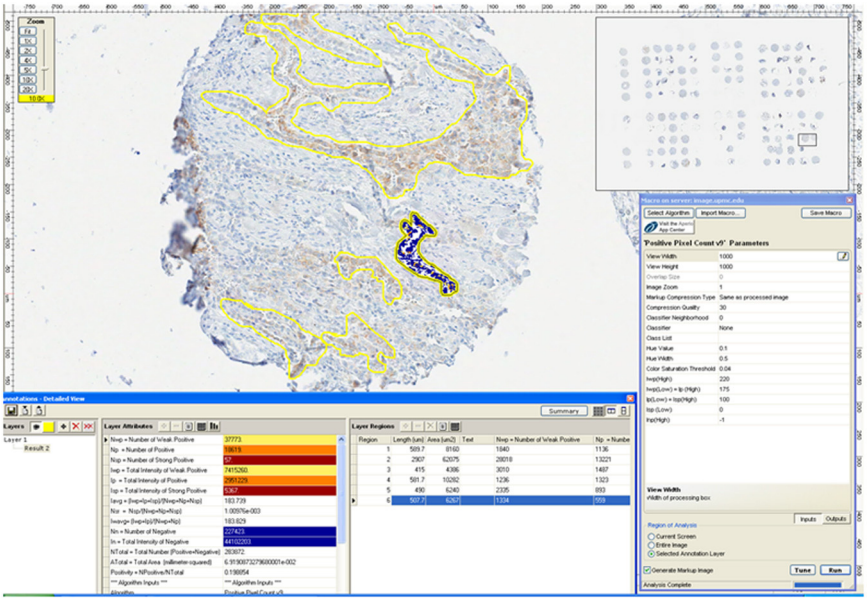

Fig. 2. Cytokeratin 5/6 immunohistochemistry showing cytoplasmic expression. Cytoplasmic algorithm output and input parameters are also seen in the background of the digital image.

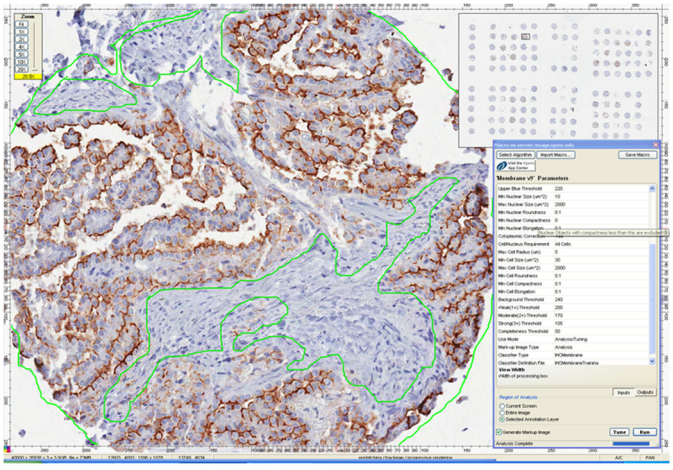

Fig. 3. D2-40 immunohistochemistry showing membrane expression. Membrane algorithm input parameters are also seen in the background of the digital image.

Results

Calretinin staining was seen in both cytoplasmic and nuclear locations. CK5/6 stain was localized to the cytoplasm. D2-40 stain showed only membranous expression in our cases. Based on the pathologist's scores, calretinin was positive in 31 of the 39 cases (80%), CK 5/6 in 15 of the 33 cases (46%), and D2-40 in 18 of the 37 cases (49%). The percent-positive agreement was 90% (35/39), 94% (31/33), and 95% (35/37) for calretinin, CK 5/6, and D2-40, respectively. There was substantial agreement between manual and automated scores for calretinin (kappa = 0.614) and almost perfect agreement for CK5/6 (kappa = 0.879) and D2-40 (kappa = 0.892) (Tables 2–5).

Table 2
Calretinin ($N = 39$): manual vs. automated scores.

Method	Image analysis: positive	Image analysis: negative
Manual: positive	31	0
Manual: negative	4	4

Table 3
CK 5/6 ($N = 33$): manual vs. automated scores.

Method	Image analysis: positive	Image analysis: negative
Manual: positive	15	0
Manual: negative	2	16

Table 4
D2-40 ($N = 37$): manual vs. automated scores.

Method	Image analysis: positive	Image analysis: negative
Manual: positive	17	1
Manual: negative	1	18

Table 5
Concordance between manual and automated scores.

Immunohistochemical stain	Percent positive agreement	Kappa statistic
Calretinin	90	0.614
CK 5/6	94	0.879
D2-40	95	0.892

Discussion

We performed immunohistochemical assessment using three commonly used mesothelioma markers (calretinin, CK5/6, and D2-40) on the TMA constructed from patients with mesothelioma at UPMC that belong to NVMB. We also compared the manual (semi-quantitative) scores with automated scores and assessed the agreement between the two methods.

The antibodies used in our study for immunohistochemical staining of calretinin, D2-40, and CK 5/6 are similar to those described in other studies [6,9,10]. As described by Husain et al. calretinin positivity was observed in both nuclear and cytoplasmic locations, and D2-40 positivity was observed along the cell membrane in our study. CK 5/6 showed cytoplasmic positivity [11].

NMVB Mesothelioma TMA slide was digitized by Aperio ScanScope XTslide scanner. The percentage of tumor cells with positive staining and the average staining intensity into positive (1+ and 2+ composite score and negative score of 0) were recorded for each biomarker. Combined scores based on the percentage of cells

stained times intensity (ranging from 0 to 300) were generated for each pathologist and then averaged. To measure the repeatability of visual scores, one pathologist rescored a random sample of 10% of the spots masked to her previous scores. Stains for calretinin, D2-40, and CK 5/6 were considered positive if the average combined score was $\geq$10.0 and negative if the average combined score was less than 10%. Semiquantitative categories described as weak, moderate or strong staining for the average pathologist scores were also created. For the positive pixel algorithm (calretinin and CK5/6), weak positive pixels were considered negative, and moderate and strongly positive pixels were considered positive.

Our results showed substantial agreement between manual and automated scores for calretinin (kappa = 0.614) and almost perfect agreement for CK5/6 (kappa = 0.879) and D2-40 (kappa = 0.892). Other studies using similar technology have also shown an excellent correlation between manual and automated scores for immunohistochemistry [12,13]. Quantification of immunohistochemistry using image analysis improves the accuracy and reproducibility of the pathologists' interpretations by eliminating inter/intra-observer variability, leading to better information for clinicians in treatment decisions for patients. This is especially important given the ongoing efforts to move away from laborious semi-quantitative (manual) scoring, with its inherent subjectivity, toward the ultimate goal of providing a standard, reproducible, sensitive and specific method of biomarker quantitation using automated analysis [14].

Conclusion

Our study confirms that the immunohistochemical staining pattern of Mesotheliomas in the NMVB UPMC TMA is similar to other studies. Our findings also show that automated image analysis provides similar results to manual scoring by pathologist, and provides a reproducible, objective, and accurate platform for immunohistochemical assessment of biomarker expression.

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