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# Validation of tissue microarray technology in malignant pleural mesothelioma

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**Summary**

*Aims:* Tissue microarray (TMA) technology has been utilised for assessment of cancers including malignant pleural mesothelioma (MPM). Given the intralesional heterogeneity of MPM, it is questionable if TMAs can adequately represent MPMs. We here investigate the validity of TMAs for MPM.

*Methods:* TMAs were constructed from at least five cores for each of 80 archival tumours processed by two centres between 1994 and 2009. The percentage of cases correctly subtyped on TMAs compared with whole sections, in relation to the number of cores analysed, was calculated. Immunohistochemical labelling for calretinin and D2-40 was performed on TMAs and whole sections. To evaluate the validity of quantitative immunohistochemistry, percentages of positive cells were recorded and two-way analysis of variance (ANOVA) performed.

*Results:* Five cores were assessable for 91% of patients. Four cores were sufficient to reach concordance with the whole-section result in 98% of cases for calretinin and 99% for D2-40. The correlation of the quantitative scores between the whole section and TMA cores was statistically significant (D2-40, rho = 0.84, $p < 2.2e\text{-}16$; calretinin, rho = 0.65, $p = 7.9e\text{-}11$). Neither the origin nor age of the blocks affected the results.

*Conclusion:* If a minimum of four cores is used, TMA is an appropriate method for immunohistochemistry in MPM.

*Key words:* Calretinin, D2-40, malignant mesothelioma, tissue microarray.

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## INTRODUCTION

Since the advent of modern tissue microarray (TMA) technology in 1998,[1] there has been an explosion of studies using this technology.[2] TMAs typically consist of arrays of small core biopsies from hundreds of formalin fixed, paraffin embedded (FFPE) tissue samples arranged in an organised fashion on a microscope slide. These can be used to quantify the expression and subcellular localisation of proteins, as well as quantify RNA levels and DNA copy numbers. The increasing use of TMA as a research tool is largely due to the high-throughput nature of the technology. It allows very small amounts of tumour from a tissue block to be examined multiple times for different molecular markers, and also allows the analysis of large numbers of samples in parallel.[1–3] The use of clinically annotated samples for TMA construction allows investigation of the relationship between biomarker expression and clinicopathological features. This technology has been applied to a variety of cancers for the investigation of diagnostic as well as molecular prognostic and/or predictive markers.[1–5]

The foremost limitation of TMA technology relates to tumour heterogeneity, and concerns have been raised as to whether the small cores assessed adequately represent the whole tumour.[1–5] There have been a number of studies that investigated the validity of TMA technology in a variety of tumour types, including ovarian, bladder, gastric, breast, colorectal cancers and soft tissue sarcoma.[6–11] Most studies confirmed that the small cores of the TMAs adequately represented the whole section for a variety of molecular markers, provided that more than one core was taken for each sample.

Although TMA technology has yet to be validated specifically in malignant pleural mesothelioma (MPM), this technique has been applied to MPM on several occasions.[12–23] The different MPM studies employing TMA reported in the literature to date did not utilise uniform technology but utilised cores of differing diameters (0.6 mm or 1.0 mm) and the number of cores varied between two and five. None of the studies validated their approach against whole sections of the tumour.

Given the significant heterogeneity of MPM and the often patchy immunolabelling of a variety of antibodies in the same section,[24] there is uncertainty whether TMA technology can provide adequate representation in MPM. This study aims to address the validity of TMA technology in MPM, using the well established diagnostic mesothelial marker calretinin and the newer and less well established marker D2-40.[12,25–27]

## MATERIALS AND METHODS

### Tumour samples and histological subtype determination

This series consisted of 80 MPM patients who underwent extrapleural pneumonectomy (EPP) at Royal Prince Alfred (RPAH) or Strathfield Private Hospital (SPH) between 1994 and 2009. The specimens were all FFPE tumour blocks, but the fixation protocol differed between the two pathology departments involved. RPAH adopted a conventional fixation protocol with

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10% buffered formalin fixation overnight at room temperature (21°C) while SPH used an accelerated protocol where specimens were fixed with 10% buffered formalin for 90 min at elevated temperature (50°C). All diagnoses of MPM and the subtypes were confirmed by a panel of experienced pathologists (DWH, SK and KL) from the whole sections. A biphasic histological subtype was assigned if both epithelioid and sarcomatoid components were present, and exceeded 10% of the cross sectional area in the slides examined.[28] This conforms to the accepted WHO criteria and means a tumour that consists of a predominantly epithelioid component with less than 10% sarcomatoid component would be classified as an epithelioid tumour.

This work was conducted as part of a larger study aimed at identifying prognostic factors in MPM and was approved by the Human Research Ethics Committee at Concord Repatriation General Hospital, Sydney, where the Asbestos Diseases Research Institute is based.

#### Tissue microarray construction

The original haematoxylin and eosin (H&E) slides from all FFPE blocks were reviewed under the microscope. The total number of blocks had not been recorded for all specimens, but where data were available ($n = 66$), the median number of FFPE blocks that resulted from each EPP procedure was 23, with a range from 12 to 57. Areas representative of the tumour on the whole section were marked and corresponding areas were then marked on the FFPE blocks.

The Advanced Tissue Arrayer, ATA-100 (Chemicon, USA) was used for construction of the TMAs. Five to six cores of 1 mm diameter were punched from the donor tumour blocks and inserted into slightly smaller holes in a recipient paraffin block, to maximise adhesion. The recipient block was arranged in an asymmetrical fashion and an accurate map of the recipient block was kept. The recipient blocks were then sectioned and H&E stain applied.

#### Immunohistochemistry

Serial 4-μm thick paraffin sections of the tumour donor blocks and the TMA blocks underwent immunohistohemical labelling for calretinin and D2-40. Primary rabbit anti-calretinin antibody (dilution 1:2000; Invitrogen, USA) and primary anti-D2-40 monoclonal antibody (dilution 1:100; Signet, USA) were applied to incubate the sections overnight at 4°C after citric acid retrieval (1:10 dilution of 0.1 M citrate buffer, pH 6.0) in a microwave. All primary antibodies were diluted in 10% normal goat serum. The reaction with calretinin was developed with the Novocastra Polymer System (Leica Biosystems, UK), using the Liquid DAB and Substrate Chromogen System (Dako, USA), whereas the reaction with D2-40 was developed with the EnVision + Dual Link System (Dako), using the DAB Substrate Kit (Cell Marque, USA).

#### Immunohistochemical evaluation

Immunohistochemical (IHC) evaluation was performed initially for the whole sections. Blinded to this result, the same observers evaluated the TMA cores.

For the diagnostic evaluation, IHC labelling was scored on an ordinal scale: positive labelling = 1; equivocal labelling = 0.5; and no labelling = 0. Only nuclear labelling was accepted as positive for calretinin, while only membranous labelling was accepted as positive for D2-40. Equivocal labelling was assigned if it was uncertain whether the labelling was genuine or just high background staining, or if <2% of tumour cells were labelled.

For the quantitative evaluation of IHC scores of calretinin and D2-40 the percentage of cells labelled by the antibodies was recorded, irrespective of the intensity, resulting in a percentage score that ranged from 0 to 100%. A score was assigned to the whole section of the donor block and a corresponding score was derived from the average of the available cores in the TMA.

#### Evaluation of the primary hypothesis

Our primary hypothesis was that TMA is a valid research tool for the assessment of MPM, despite the known intralesional heterogeneity of the tumour. The diagnostic antibodies of calretinin and D2-40 were used to address this hypothesis. Questions related to the hypothesis included:

1. Is TMA a valid tool for the diagnostic evaluation of MPM by IHC?
2. How many cores per tumour are necessary to adequately represent the tumour?
3. Are the quantitative IHC scores comparable between the whole section and the average of the cores from the TMA?
4. Is there a difference in protein expression between the specimens from the two pathology centres, in view of the differing fixation protocols?
5. Does the age of the FFPE blocks impact on the ability to detect expressed protein, i.e., is there degradation of protein?
6. What is the intra-tumoural heterogeneity (between cores)?

These questions were addressed by the following statistical methods. Data consisted of the diagnostic ordinal scales and the quantitative IHC scores of calretinin and D2-40 for up to six cores from 80 patients as well as the whole section. Histological subtype was also assessed for each core and the whole section. Patient information included the centre that processed the samples (15 patients from RPAH versus 65 patients from SPH) and the year of diagnosis (36 patients from years 1994–2003 versus 44 patients from years 2004–2009).

From the diagnostic IHC assessment point of view, we considered clear positive labelling as necessary to interpret the result as positive in clinical practice. Therefore, equivocal labelling was considered negative for the purpose of analysis in this study. The IHC labelling of calretinin and D2-40 was considered concordant between the whole sections and the TMAs, if at least one core exhibited a concordant result with the whole section. The percentage of cases correctly classified (positive versus negative labelling of calretinin and D2-40) on TMA, in comparison with the whole section, in relation to the number of cores analysed by case, was calculated. This took into account technical problems such as missing cores as well as discordant results.

Quantitative scores from the whole sections were compared to the average scores from the cores using a simple Pearson correlation. The impact on the average TMA scores of the different centres involved in the study, as well as the year of diagnosis, was assessed by two-way analysis of variance (ANOVA), taking into account the fact that the histological subtype of the tumours (assessed from the whole section) may affect the IHC scores independently of centre/year. Finally, reproducibility of the TMA scores was determined from the differences between the five cores and primarily expressed as an intraclass correlation coefficient (ICC).[29] All statistical analysis was carried out using the R software package (R Foundation for Statistical Computing, Austria; http://www.r-project.org). One-way ICC was calculated using the irr library in R.

## RESULTS

### Diagnostic evaluation

*Histological subtype*

Of the 80 patients, 61 had an epithelioid MPM (76%) and 19 had a biphasic subtype (24%) as assessed from the whole sections from the donor blocks.

The median number of cores that contained assessable tumour on H&E stains was five (range 0–6 cores): one case yielded no assessable tumour on the TMA (1.3%); one case yielded two assessable cores (1.3%); two cases yielded three assessable cores (2.5%); three cases provided four assessable cores (3.8%); 64 cases had five cores (80%) available for assessment; and nine cases provided six assessable cores (11%).

Table 1 details the range of histological subtypes as assessed on the individual cores compared to the subtype determined on the whole section.

*Immunohistochemistry*

From the donor tumour blocks, 78 cases exhibited convincingly positive (1) nuclear labelling for calretinin (98%), one case had equivocal (0.5) labelling while one had negative (0) labelling for calretinin.

An overall assessment of the TMAs revealed that 78 cases (98%) showed concordant calretinin labelling in at least one core. The two discordant cases were a case with equivocal labelling on the whole section and all five cores positive; and a case with no assessable tumour on the TMA and therefore no assessment could be made.

From the donor tumour blocks, all 80 cases (100%) demonstrated positive (1) membranous labelling for D2-40.

**Table 1** Histological subtype

| Histological subtype on whole section | Histological subtype on the individual tissue microarray cores | | | | | | |
|---|---|---|---|---|---|---|---|
| | All epithelioid | All biphasic | Mixture of epithelioid/biphasic | Mixture of epithelioid/sarcomatoid | Mixture of biphasic/sarcomatoid | Mixture of epithelioid/biphasic/sarcomatoid | No tumour |
| Epithelioid ($n=61$) | 43 | 0 | 15 | 0 | 0 | 2 | 1 |
| Biphasic ($n=19$) | 1 | 1 | 8 | 4 | 4 | 1 | 0 |

Correspondingly, all cases from TMA had at least one core that demonstrated positive labelling for D2-40.

The percentage of cases that had concordant diagnostic IHC evaluation in TMA, in comparison with the whole section, in relation to the number of cores analysed by case, is shown in Table 2.

### Evaluation of quantitative IHC scores

The median calretinin score was 60% on the whole section [range 0–100%; standard deviation (SD) 26.7] while the median calretinin score was 48% for the TMA (range 0–97%; SD 26.6). The median D2-40 score was 50% on the whole section (range 2–95%; SD 27.6) while the median D2-40 TMA score was also 50% (range 2–96%; SD 30.0).

The correlation of the quantitative scores between the whole section and the average of the cores was calculated. In both cases, the correlation was found to be statistically significant and was strongly positive (D2-40 rho = 0.84, $p < 2.2e\text{-}16$; calretinin rho = 0.65, $p = 7.9e\text{-}11$). The strong association can be seen clearly in the scatter plots in Fig. 1.

### Quantitative IHC scores between the two pathology centres and the two time periods

For both calretinin and D2-40, the difference in the quantitative scores between the two centres was not statistically significant (calretinin $p = 0.52$; D2-40 $p = 0.13$). Similarly, neither calretinin ($p = 0.15$) nor D2-40 ($p = 0.18$) quantitative scores were found to be significantly different between the two time periods.

### Intratumoural heterogeneity

The ICC for calretinin ($n = 69$) was 0.786 [95% confidence interval (CI) 0.715–0.848), while the ICC for D2-40 ($n = 73$) was 0.873 (95%CI 0.828–0.911). This indicated that the cores for the individual patients were in strong agreement.

## DISCUSSION

The validity of TMA technology in MPM was addressed in this study of a retrospective series of 80 patients who underwent EPP, using the two diagnostic mesothelial markers, calretinin and D2-40. These antibodies were selected for this validation study for two reasons. Both antibodies have a well established role in the diagnosis of MPM, with calretinin having been used for longer and with D2-40 being a more recent antibody which, in our view, has proved useful for diagnosis. Importantly, both antibodies label different parts of the tumour cells, with calretinin labelling the nucleus of MPM cells and D2-40 exhibiting membranous labelling. Furthermore, for both these antigens, expression within the individual MPM has been described as significantly heterogeneous.[12,24]

From the diagnostic evaluation point of view, the results demonstrate that the histological subtype cannot be accurately assessed from the 1 mm cores as it is subject to high sampling error rate due to the small diameter of the cores. Even though the areas representative of the tumour were marked and all efforts were made to match the block for the construction of the TMAs, there were discrepant findings in the histological subtype in the individual cores compared to the whole sections. In part, this is probably due to the fact that the marking of the tumour was performed in the original H&E slides and with subsequent tissue handling and deeper cutting having been performed, the tumour faces in some cases were different. In the much smaller amount of tissue sampled in the TMAs compared to whole sections, a small change in relative proportion of cell type would result in exaggerated alteration

**Table 2** Percentage of cases that had the concordant immunohistochemistry evaluation on tissue microarray, in comparison with the whole section, in relation to the number of cores analysed by case

| Antibody | Number of cores punched with concordant staining by case | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| Calretinin ($n=80$) | 91% | 94% | 95% | 98% | 98% |
| D2-40 ($n=80$) | 91% | 96% | 99% | 99% | 100% |

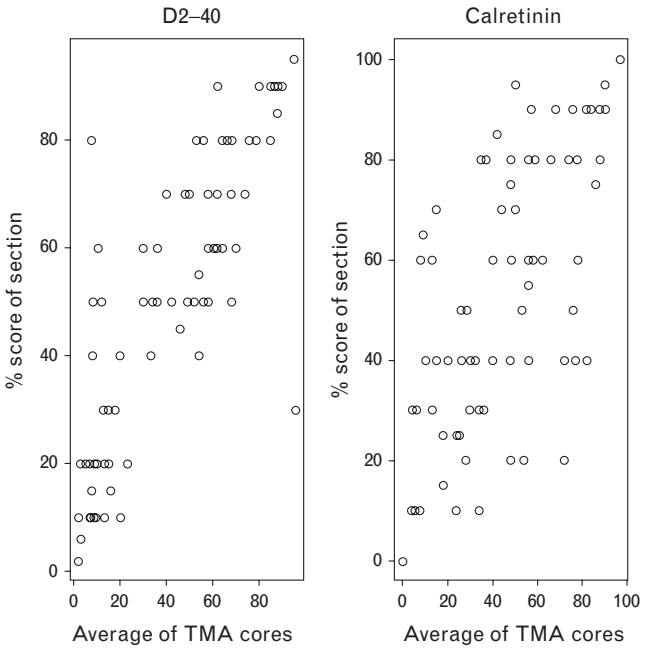


**Fig. 1** Scatter plots demonstrating the relationship of the quantitative immunohistochemistry scores between the whole section and the average of the tissue microarray cores for D2-40 and calretinin antibodies. TMA, tissue microarray.

in the relative distributions of epithelial and sarcomatoid cell types.

As mentioned previously, to diagnose the biphasic subtype of MPM, the International Mesothelioma Panel arbitrarily recommended the presence of at least 10% of both an epithelioid or a sarcomatoid component in the sample.[28] This means a tumour that consists of a predominantly epithelioid component with less than 10% sarcomatoid component would be classified as an epithelioid tumour. Therefore, the gold standard of assigning a histological subtype requires the use of a whole section.

This is supported by previous findings, since it has been shown that biopsy size is important for accurate diagnosis of MPM, and that bigger biopsies yield better and more reliable results.[30,31] Attanoos and Gibbs found that in specimens $<10$ mm in size, definitive diagnosis of MPM (regardless of histological subtype) was only attained in 8% of cases.[30]

This study also demonstrates that a minimum of four cores is required to achieve a sufficiently high sensitivity with concordant diagnostic IHC labelling. Using four cores, the TMA correctly classified the calretinin labelling in 98% of cases, while it classified the D2-40 labelling correctly in 99% of cases. An additional core did not increase the sensitivity significantly with correct calretinin labelling remaining at 98% and D2-40 labelling increasing to 100%. This took into account false negative labelling due to sampling error as well as technical issues such as the lack of assessable cores. This result is in agreement with the recent review by Pintilie *et al.* who suggested that three to five cores per patient are necessary to overcome the problem of tumour heterogeneity in the conduct of a well designed biomarker discovery study.[32]

The quantitative assessment of protein expression by IHC remains controversial and there is currently no consensus of the optimal scoring system for any new biomarkers. Some utilise either the intensity or the percentage of cells labelling alone, while others use a semi-quantitative score with multiplication of both the intensity and the percentage. However, a semi-quantitative form of assessment is necessary to examine the impact of protein expression on the clinical outcome. In this study, the decision not to take into account the intensity score was based on several reasons. From our previous experience, intensity can be extremely subjective and several factors can alter the outcome, such as uneven thickness of the section created by differing sharpness of the blade of the microtome, subtle inconsistencies of manual incubation times between different batches of the experiments, and degree of background staining.

Using percentage as the quantitative IHC score, both the calretinin and D2-40 showed strong agreement between the whole sections and the mean of the TMA cores. This study also confirmed the large interpatient variability of calretinin and D2-40 protein expression as indicated by the wide standard deviation for both antibodies. Interestingly, the differences in protein expressions between the cores expressed as an ICC in statistical terms demonstrated that the cores had strong agreement for each patient, indicating that intra-tumoural heterogeneity was not as much of a problem as initially expected.

For a biomarker discovery study to be clinically useful, a large enough cohort of patients with clinico-pathological and treatment parameters and overall survival is required to demonstrate the prognostic or predictive role of a biomarker with sufficient statistical power. In a relatively uncommon tumour like MPM, it may require multi-institutional input (with potentially differing fixation methods) and/or years of recruitment. This has led to concern regarding the role of differing fixation methods and the age of the FFPE blocks affecting the antigen survival, hence adding bias to the results. There is some evidence to suggest that tissues retain their antigenicity despite being stored as FFPE blocks for up to 70 years.[9] Our study results demonstrated that there were no statistical differences in protein expression between the two pathology centres that processed the samples or based on the age of the blocks. This provides the reassurance that the antigen durability is not significantly affected by differing fixation methods or the age of the blocks in MPM.

In conclusion, we demonstrated that TMA is a valid research tool in the investigation of MPM. With the exception of the assessment of histological subtype, TMAs appear to adequately represent a tumour's immunohistochemical profile—both the assessment of diagnostic IHC labelling, as well as the quantitative IHC scores—provided that a minimum of four cores are sampled per patient. This work will serve as the rationale for our group and others to proceed with further TMA constructions in MPM, thereby increasing the power of subsequent studies of potential prognostic and predictive markers.

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