Tissue Microarray Technique in Evaluation of Proliferative Activity in Invasive Ductal Breast Cancer

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Abstract. *Aim: Investigation of the relationship between expression of Ki-67 antigen and minichromosome maintenance 2 (MCM-2) protein by using an immunohistochemical study on whole sections (WS) and sections of invasive ductal breast cancer (IDC) obtained in the tissue microarray (TMA) technique. Materials and Methods: Material consisted of 51 archival paraffin blocks of IDC. Tissue microarrays were composed of 0.6 mm core punches. Reactions were performed using specific antibodies (anti-Ki-67 and anti-MCM-2). Intensity of the marker expression was evaluated using computer-assisted image analysis. For statistical purposes, three different tests were used. Results: Spearman rank correlation revealed a strong positive correlation between expression of tested markers: Ki-67 (TMA) vs. Ki-67 (WS) ($r=0.91$, $p<0.05$) and MCM-2 (TMA) vs. MCM-2 (WS) ($r=0.87$, $p<0.05$). Mann Whitney U-test showed no significant differences between the two markers in both analysed techniques. Moreover, the Bland–Altman plot demonstrated a low level of bias between the analysed methods. Conclusion: This study shows that TMA could have a great potential in evaluation of proliferative activity in IDC and that MCM-2 protein might be a specific and sensitive marker of cell proliferation.*

Tissue microarray (TMA) technology was described by Wan *et al*. in 1987 (1). They published a modification of an idea of H. Battifora in 1986 of a so–called "multitumor (sausage) tissue block" (2). In 1998, J. Kononen and collaborators developed a device that was able to rapidly and reproducibly produce TMAs (3).

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TMA is a method of re-locating tissue from conventional paraffin blocks. In its most common form, a core of tissue is lifted from a formalin-fixed, paraffin-embedded sample and placed in predrilled hole in recipient paraffin block. On sectioning, each sample is represented as a small (0.6- to 2.0-mm in diameter) histological section (histospot) arrayed in a grid that allows easy linkage to clinicopathological data. The result is a single slide that contains samples from 40 to 800 patients (depending on core size). A newer alternative, the cutting edge matrix assembly array, is produced by cutting and stacking sections in a serial manner to produce arrays that represent thousands of specimens. Other researchers have adapted TMA technology in the study of frozen tissues, cell lines, and needle biopsies (4-6).

Ki-67 is widely used as a proliferation marker but its value as a prognostic marker has not been sufficiently investigated. Many studies tested the relationship between cell proliferation and the clinical course of a disease, often yielding divergent results (7-13). Although Ki-67 is used routinely, its biological function remains unknown. There are some hypotheses concerning its function, which is probably not linked to any key elements of the cell proliferation mechanism (14). Expression of Ki-67 may also be observed in situations of inhibited DNA synthesis or apoptosis (15). Since the biological function of this protein, as well as its significance as a prognostic factor are unclear, it has been proposed that other proliferation markers, such as minichromosome maintenance 2 (MCM-2) protein, may be useful in clinical and pathological examination. The MCM represents a family of homogenous proteins, six of which (MCM-2 to 7) are mainly responsible for the commencement and maintenance of replication (16, 17). At the early G_1 phase during the cell cycle, MCM proteins compose the pre-replication complex, which, due to their helicase activity, allow them to disentangle DNA threads during replication process (18, 19). Moreover, MCM-2 irreversibly binds to chromatin warranting that only one genetic material replication event occurs per

single cell division (20, 21). In view of the presented facts, expression of MCM family proteins, including MCM-2, may be the most frequently applicable marker for clinical and pathological purposes in the future, especially for tracking rapidly dividing cells (22-28).

Most studies assay biomarkers on TMAs using immunohistochemical (IHC) techniques, but to our knowledge we are the first to test for reproducibility and accuracy of proliferation marker assessment on TMAs as compared to traditional assessment in whole tissue sections.

Materials and Methods

Patient samples. The studies were performed on 51 archival paraffin blocks containing fragments of invasive ductal breast cancer (IDC), sampled in 2000 during procedures of mammectomy in the Lower Silesian Oncology Centre in Wroclaw. All the patients were female and the grade of malignancy (G) was G1 in 10 cases, G2 in 30 cases and G3 in 11 cases.

TMA construction and tissue samples. Tissue samples were fixed in 10% buffered formalin. Subsequently they were dehydratated and embedded in paraffin blocks. For the construction of the TMA blocks, a new section was made from paraffin donor block and stained with hematoxylin-eosin (HE). Two additional sections were cut for performing IHC on whole tissue sections. The HE sections were examined by two independent pathologists (CK, BP) under light microscopy (BX-42; Olympus, Tokyo, Japan) and areas of interest were circled using a permanent marker. From the corresponding paraffin blocks, three 0.6 mm core punches were taken for each case using a Manual Tissue Arrayer I (Beecher Instruments Inc, Sun Prairie, Wisconsin, USA) and transferred into the recipient paraffin block.

IHC. Immunohistochemical reactions were performed on paraffin sections (4 μm) mounted on Superfrost Plus slides (Menzel Gläser, Braunschweig, Germany) cut from whole tissue and TMA paraffin blocks. Deparaffinization and antigen retrieval were performed in Target Retrieval Solution, pH 6 (97˚C, 20 min) and PT Link Rinse Station. The sections were then washed in Tris-buffered saline (TBS) and incubated with primary antibodies: anti-Ki-67 (MIB-1, 1:100; Dako Cytomation, Glostrup, Denmark) and anti-MCM-2 (CRCT2.1, 1:50; Novocastra Laboratories, Newcastle, UK) in a Link48 Autostainer (room temperature, 20 min). EnVision FLEX was used for the visualization of the antigens, in accordance with the manufacturer's instructions. All slides were counterstained with Mayer's haematoxylin. All the antibodies, reagents and equipment except for the anti-MCM2 antibody were obtained from Dako Cytomation.

Evaluation of the intensity of IHC reaction. For the evaluation of Ki-67 and MCM-2 in each paraffin section and TMA core three fields with the highest number of tumour cells yielding a positive reaction were selected (hot spots). The percentage of positive cells in each hot spot was evaluated under ×400 magnification, scoring the brown-labeled cell nuclei of cancer cells (BX-42 light microscope equipped with Cell^D software for computer-assisted image analysis; Tokyo, Japan). The general result for every sample was an average of the three hot spot percentages. The intensity of the IHC reactions in coded preparations were independently evaluated by two pathologists (CK, BP). Moreover in doubtful cases, a re-evaluation with a double-headed microscope was performed until a consensus was achieved.

Table I. *Correlation between investigated proliferation markers (Ki-67 and minichromosome maintenance 2 (MCM-2) protein) assessed in whole tissue sections (WS) and tissue microarrays (TMA).*

	Ki-67 (WS)	Ki-67 (TMA)	MCM-2 (WS)	MCM-2 (TMA)	Tumour grade
Ki-67 (WS)	X	0.91 $p<0.05$	0.60 $p<0.05$	0.59 $p<0.05$	0.51 $p<0.05$
Ki-67 (TMA)		X	0.65 $p<0.05$	0.65 $p<0.05$	0.50 $p<0.05$
MCM-2 (WS)			X	0.87 $p<0.05$	0.38 $p<0.05$
MCM-2 (TMA)				X	0.44 $p<0.05$
Tumour grade					X

Statistical analysis. The results were subjected to statistical analysis using Statistica 7.1 PL and Prism 5.0 (Statsoft, Krakow, Poland and GraphPad, La Jolla, CA, US, respectively). The correlation between the expression of both markers and grade of malignancy was examined using Spearman's rank correlation test. Mann–Whitney *U*-test and Bland-Altman test were used to compare the results from whole tissue sections and TMAs (29, 30). In all analyses, results were considered to be statistically significant when $p<0.05$.

Results

The majority of the examined tumours manifested nuclear expression of Ki-67 antigen and MCM-2 protein (Figure 1). Statistical analysis of the relationship between expression of the same marker on whole sections (WS) and TMA demonstrated a strong positive correlation for Ki-67 antigen: Ki-67 (TMA) *vs*. Ki-67 (WS) (r=0.91, $p<0.05$) and for MCM-2 protein: MCM-2 (TMA) *vs*. MCM-2 (WS) (r=0.87, $p<0.05$). Statistical analysis also revealed positive, significant correlations of both analysed antigens in whole tissue sections, as well as in TMAs, with the grade of malignancy. These results are summarized in Table I. Mann Whitney U-test showed no significant differences between the percentage of positive cells in whole tissue sections and TMAs regarding Ki-67 and MCM-2 expression (Figure 2A and B). Similar results were noted for both antigens regarding the grade of malignancy, when scores for each antigen were analysed within the same grade (Figure 2C and D). Bland–Altman test revealed a low level of bias between analysed tissue specimens for both Ki-67 antigen and MCM-2. Standard deviation of bias for Ki-67 (WS) *vs*. Ki-67 (TMA) was 9.52%, whereas for MCM-2 (WS) *vs*. MCM-2 (TMA) it was 11.67%.

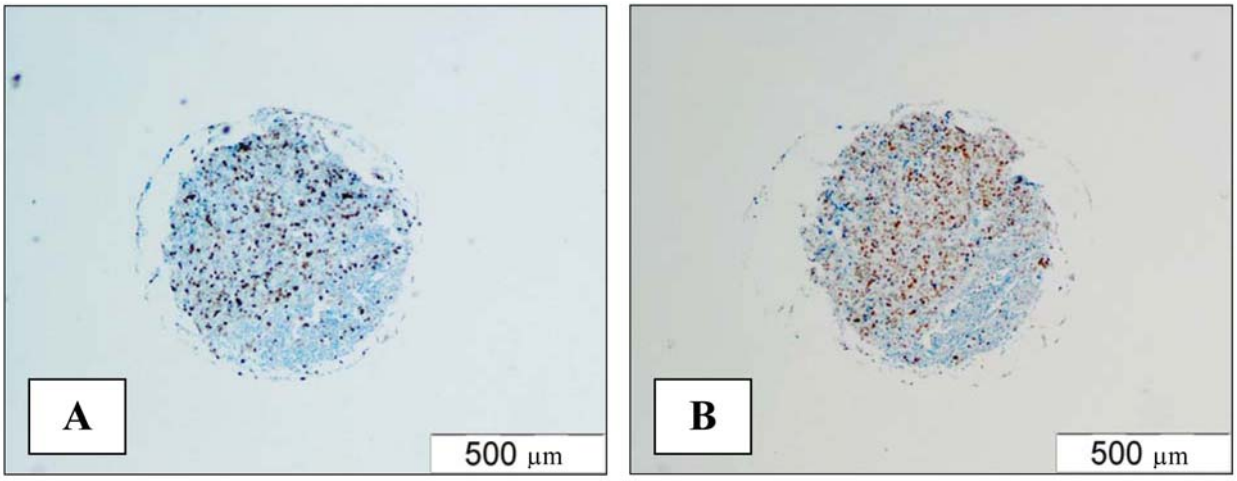

Figure 1. *Expression of Ki-67 antigen (A) and minichromosome maintenance 2 (MCM-2) protein (B) in TMA cores of invasive ductal breast cancer.*

Discussion

Development of hardware for TMA preparation, is essential, since the interest in this diagnostic and research tool has increased and has recently become very popular. TMAs in cancer research give the opportunity to analyse the frequency of a molecular alteration in different tumour types, evaluate prognostic markers, test potential diagnostic markers and optimize antibody staining conditions. Research teams continue to investigate the advantages, disadvantages and possibilities of using this method. Camp *et al*. reviewed the literature describing the decade of development of TMA (31).

Depending on the spectrum of scientific research, different tissues are examined using TMA. Most research in this field was conducted to compare the expression of specific markers on whole sections and TMA. In our study, we analysed the expression of Ki-67 antigen and MCM-2 protein in a group of IDC cases. We examined the correlation between these proliferation markers in whole tissue sections and in TMAs. Spearman correlation showed a strong positive correlation between these proteins in whole tissue sections and as well as in TMAs. Additionally, the positive correlations of both Ki-67 and MCM-2 expression with the grade of malignancy were observed. In our previous studies, we have shown that Ki-67 strongly correlates with MCM-2 expression in breast cancer on classical whole tissue sections (28). This study confirms our previous observations.

An important argument against the use of TMA is the heterogeneity of cancer tissues. Kyndi *et al*. carried out IHC staining for estrogen receptor, progesterone receptor and human epidermal growth factor receptor 2 which showed a substantial agreement between a single 1 mm TMA core and the corresponding whole section. Moreover, similar agreement was shown between the central and peripheral cores, and between cores from two different paraffin blocks from the same tumour (32). In conclusion, the authors stated that the use of TMA cores seemed to be sufficient and no significant heterogeneity was noticed. Similarly, we have demonstrated no significant differences between the expression of Ki-67 and MCM-2 analysed in whole tissue sections and TMAs. To minimize the problem of tissue heterogeneity, Bolton *et al*. suggested the use of automated image analysis for tissue scoring (33). The researchers assessed agreement between the automated and the pathologist as score of a diverse set of IHC assays performed on breast cancer TMAs. The agreement was found to be excellent. They suggested usage of this method for epidemiological investigations (33). Although in our research we have also used computer–assisted image analysis, it served only for standardization purposes.

An important aspect of the TMA is also the diameter of the punch. A well-known fact is that pathologists frequently feel more confident of the results if larger core size is used. There is no evidence in the literature showing that large cores are better nor worse than small cores for assessment of TMAs. Anagnostou *et al*. conducted experiments with different sizes of cores and found that TMAs with 0.6 mm cores are as representative as those with any common larger sized core (34). Karlsson *et al*. conducted comparative studies of 0.6 and 1 mm cores on breast, lung and endometrial cancer tissue and found that both core sizes were equally informative regarding Ki-67 expression (35).

Studies were also conducted on TMA testing specimens obtained from regular needle biopsy. In their work, Vogel and Bültmann suggested that biopsy material may be used for TMA construction without proper equipment in order to reduce costs and could be considered as an optimal solution in pathological practice (36). Moreover, Munirah *et al*. stated that TMA may provide easy access to similar samples examined by the

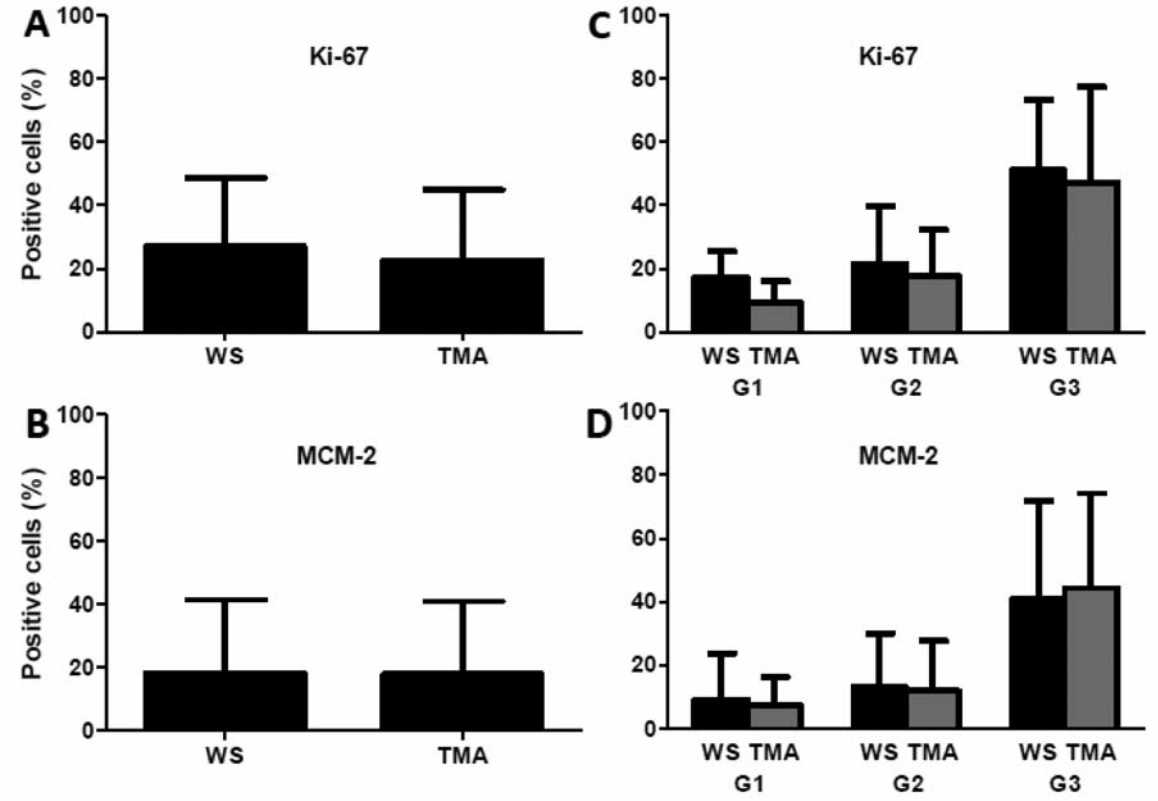

Figure 2. *Expression of Ki-67 antigen (A, C) and minichromosome maintenance 2 (MCM-2) protein (B, D) in whole tissue sections (WS) and TMA sections overall (A, B) and regarding the grade of malignancy (C, D).*

pathologist and therefore improving the repeatability of the results (37). Furthermore, comparison of TMA with whole tissue sections may be proposed as an internal laboratory control for improving the credibility of this method.

In conclusion, through our study we have shown that TMA is a reliable technique for examining a large set of tumours. We validated this technique in breast cancer specimens by comparing the IHC staining results obtained for proliferation markers Ki-67 and MCM-2 in TMA with those from classical whole tissue sections. As no significant differences were observed between the two methods, we suggest that TMA may be useful for a wide spectrum of histological examination of various tissue preparations and IHC markers.

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