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Tissue microarrays for comparing molecular features with proliferation activity in breast cancer

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Tissue microarrays (TMAs) are potentially suited to find associations between molecular features and clinical outcome. Enhanced cell proliferation, as measured by Ki67 immunohistochemistry, is related to poor patient prognosis in many different tumor types. Ki67 expression shows considerable intratumoral heterogeneity. It is unclear if the TMA format is suitable for the analysis of potentially heterogeneous markers because of the small size of TMA spots. We have analyzed a breast cancer TMA containing 2,517 breast tissues, including 2,222 neoplastic and 295 normal or premalignant samples, for Ki67 labeling index (Ki67 LI) and additional markers with a known relationship to Ki67 LI by immunohistochemistry (ER, PR, Bcl-2, Egfr, p16, p53) and Fluorescence *in situ* hybridization (HER2, MDM2, CCND1, MYC). A high Ki67 LI was linked to tumor phenotype including grade ($p < 0.0001$), stage ($p < 0.0001$), nodal stage ($p = 0.0018$), and patient prognosis ($p < 0.0001$), elevated protein levels of p53, p16 and Egfr, reduced levels of Bcl2, ER, and PR ($p < 0.0001$ each), as well as amplifications of HER2, MYC, CCND1 and MDM2 ($p < 0.0001$ each). In summary, all expected associations between Ki67 and the analyzed molecular markers could be reproduced with high statistical significance using a TMA containing only one tissue sample per tumor, measuring 0.6 mm in diameter. We conclude that associations with cell proliferation can be reliably analyzed in a TMA format.
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Key words: Ki67; breast cancer; proliferation marker; tissue microarrays

Rapid tumor cell proliferation is a critical feature for tumor aggressiveness. Many genes involved in cancer biology directly impact cell proliferation. A significant *in vivo* association with cell proliferation argues for the potential importance of a newly detected molecular feature in cancer. Studies comparing molecular markers with tumor cell proliferation are therefore frequently undertaken.[1–3]

Studies designed to show an *in vivo* impact of certain gene products on tumor cell proliferation will require a large number of cases, as tumor cell proliferation is influenced by many different factors. The tissue microarray (TMA) technology would therefore be potentially useful for this purpose.[4] In this technology, up to 1,000 tissue samples, measuring 0.6 mm in diameter can be analyzed on 1 microscope glass slide. Because of the small volume of tissues analyzed per tumor, the representativity of arrayed samples has initially been questioned. However, a variety of studies have shown that highly representative data can be obtained on TMAs.[5–7] The Ki67 labeling index (Ki67 LI) belongs to these molecular features for which many investigators would doubt the utility of the TMA approach. The Ki67 protein is expressed in all cells of the G1, S and G2/M phase of the cell cycle.[8] Determination of the percentage of Ki67 expressing cells (Ki67 LI) has become a standard procedure to assess the proliferative activity of cells on tissue sections.[9] However, it is recognized that the Ki67 expression pattern shows considerable heterogeneity between different tumor areas.

Theoretically, this would make the determination of the Ki67 labeling index (Ki67 LI) on TMAs difficult. Therefore, some authors have suggested the use of multiple cores per tumor for Ki67 analysis on a TMA.[10] However, in a recent bladder cancer study, we had found all expected associations between Ki67 LI and clinicopathological parameters in bladder cancer TMAs, independent of the number of analyzed cores.[11]

Because of the high potential utility of TMAs for analyzing the impact of molecular features on proliferative activity, we aimed to further address this question in a large scale study. Ten different biomarkers (HER2, MDM2, Egfr, MYC, CCND1, ER, PR, p53, p16, bcl2), many of which previously suspected to be related to proliferative activity in breast cancer or other tumors, were analyzed on a breast cancer TMA composed of more than 2,000 breast cancers with clinical follow-up data. The results strongly suggest that TMAs are highly useful to study the relationship between molecular features and tumor cell proliferation.

Material and methods

Breast cancer tissue microarray

A total of 2,222 formalin-fixed (buffered neutral aqueous 4% solution), paraffin-embedded tumors were available in the Institute of Pathology, University Hospital Basel, the Institute for Clinical Pathology in Basel and the Triemli Hospital in Zürich. The use of these specimens and data in research were approved by the Ethics Committee of the Basel University Hospital. The median patient age was 62 (range, 26–101) years. Raw survival data were either obtained from the cancer registry of Basel or collected from the patients visiting the physicians. The mean follow-up time was 68 months (range, 1–176). The pathologic stage, tumor diameter and nodal status were obtained from the primary pathology reports.[12] All slides from all tumors were reviewed by 1 of 2 pathologists (J.T., G.S.) to define the histologic grade according to Elston and Ellis (BRE)[13] and the histologic tumor type. The TMA composition is given in Table I. TMA construction was as described.[14] Briefly, tissue cylinders with a diameter of 0.6 mm were punched from representative tumor areas of a "donor" tissue block using a home-made, semiautomatic robotic precision instrument and brought into 6 different recipient paraffin blocks, each containing between 342 and 522 individual samples. Four micrometer sections of the resulting multitumor TMA blocks were transferred to an adhesive coated slide system (Instrumedics, Hackensack, NJ).

Fluorescence in situ *hybridization*

A set of 4-µm TMA sections was used for two-color FISH. For proteolytic slide pretreatment, a commercial kit was used (Paraffin pretreatment reagent kit, Vysis, Downers Grove, IL). A Spectrum-

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TABLE 1 – CLINICAL DATA[1]

Breast tissue type	Histology	On array	Interpretable
	All samples	2517	1965
Normal/ premalignant	Normal tissue	109	42
	Apocrine metaplasia	14	11
	Intraductal hyperplasia	27	22
	Atypical intraductal hyperplasia	5	3
	Mastopathy	22	18
	Papilloma	31	28
	Sclerosing adenosis	15	14
	DCIS	62	55
	LCIS	10	6
Cancer	Ductal carcinoma	1552	1228
	Lobular carcinoma	312	253
	Medullary carcinoma	58	52
	Tubular carcinoma	56	40
	Cribriform carcinoma	65	46
	Mucinous carcinoma	69	59
	Papillary carcinoma	30	25
	Apocrine carcinoma	15	13
	Clear cell carcinoma	14	12
	Other subtypes	29	25
	Unknown	22	13
Tumor stage	pT1	823	611
	pT2	1023	831
	pT3	124	108
	pT4	242	207
Nodal status	pN0	952	741
	pN1	794	640
	pN2	121	103
BRE grade	G1	579	442
	G2	929	744
	G3	714	579

[1]Numbers do not add up to 2,222 in tumor stage and nodal status because of missing data in a small fraction of cases.

TABLE II – ANTIBODIES, STAINING CONDITIONS AND SCORING CRITERIA

Antibodies and staining conditions

Antibody	Source	Protocol details
Ki67[4]	DAKO, Ki 67 (MIB-1; M7240)	PC[1] (120/5)[2]; 1:50
ER[5]	Novocastra, NCL-L-ER-6F11	PC (120/12); 1:1000
PR[5]	Novocastra, NCL-L-PGR-312	PC (120/6); 1:1000
P53	DAKO, M7001	MC[3] (80/30); 1:200
P16	MTM, E6H4	PC (120/5); 1:200
Bcl2	DAKO, M0887	PC (120/5); 1:200
EGFR	Novocastra (NCL-EGFR)	MC (90/80); 1:40

Scoring criteria for P53, P16, Bcl2 and EGFR

IHC result	Staining intensity	Fraction of stained tumor cells (%)
Negative	No staining	
Weak positive[4]	1+	≤50
	2+	≤20
Moderate positive	1+	>50
	2+	>20 but ≤70
	3+	≤30
Strong positive	2+	>70
	3+	>30

[1]PC: Pressure cooker.–[2](x/y): x = temperature (°C), y = heating time.–[3]MC: Microwave.–[4]Scoring as described in the text.–[5]Scored positive if staining in ≥10% of the cells.

Orange labeled gene specific probe was used along with a Spectrum-Green-labeled probe of the respective centromere as a reference. The used probe combinations were: HER2/Centromere 17 (PathVysion; Vysis), MDM2 (SpectrumOrange; provided by Vysis)/centromere 12 (CEP 12 SpectrumGreen; Vysis), CCND1 (LSI Cyclin D1 SpectrumOrange)/centromere 11 (CEP 11 SpectrumGreen; Vysis) and MYC (LSI c-myc SpectrumOrange; Vysis)/centromere 8 (CEP 8Z2 SpectrumGreen;Vysis). Before hybridization, TMA sections were deparaffinized, air-dried and dehydrated in 70, 85 and 100% ethanol followed by denaturation for 5 min at 74°C in 70% formamide-2× SSC solution. After overnight hybridization at 37°C in a humidified chamber, slides were washed and counterstained with 0.2-μM DAPI in an antifade solution. For each tumor, the average gene and centromere copy numbers were estimated. A tumor was considered amplified if the ratio of oncogene/centromere was ≥2.0.

Immunohistochemistry

Standard indirect immunoperoxidase procedures were used for the detection of Ki67 protein, p53, bcl2 (DAKO, Glostrup, Denmark), p16 (MTM Laboratories AG, Germany), estrogen receptor (Egfr) and progesterone receptor (Novocastra Laboratories, Newcastle-upon-Tyne, UK). The antibodies, their dilutions, scoring criteria, slide pretreatment and incubation details are summarized in Table II. Diaminobenzidine was used as a chromogen. Tumors with known positivity were used as positive controls. The primary antibody was omitted for negative controls. All slides were read manually. The Ki67 LI was defined as the fraction of tumor cells showing any nuclear Ki67 immunoreactivity. Tumor cells (100) were counted per tissue spot to determine the Ki67 LI. For all other antibodies the percentage of positive cells and the staining intensity (score, 1–3) was recorded for each sample. Stainings were then categorized as described in Table II.

Statistics

T-tests and analysis of variance tests were used to study the relationship between molecular features, grade, stage, nodal status and Ki67 LI. To investigate the effect of Ki67 LI on patient prognosis, survival curves were plotted according to Kaplan–Meier, and a log rank test was applied to examine the relationship between molecular or histological data and raw survival.

Results

Ki67 LI

The Ki67 LI could be determined in 1,965 of 2,517 samples of the TMA. Reasons for noninterpretable cases were missing tissue or absence of tumor cells on arrayed spots. Since inappropriate fixation conditions are known to cause false negative Ki67 immunostaining results,[15] we have excluded 57 cases with lack of Ki67 immunostaining, since they might represent tissues with reduced immunoreactivity. The mean Ki67 LI was 25.6% (range, 1–80%). Representative images of tumors with high and low Ki67 LI are shown in Figure 1. For the purpose of determining the impact of the Ki67 LI on patient survival, tumors were categorized into 4 similar large groups (very low, low, intermediate, high proliferation). The cutoffs were selected as follows: very low 1–14%, low 15–23%, intermediate 24–34% and high >34%.

Relationship with clinicopathological parameters

The Ki67 LI was significantly associated with all clinicopathological parameters analyzed (Table III). This was particularly true for the BRE grade. The Ki67 not only increased significantly with the BRE grade but also with its individual components (polymorphy, tubulus formation, mitoses; $p < 0.0001$ each). Also, there was an almost linear increase of the Ki67 LI with the BRE score (Fig. 2). Ki67 LI was also associated with pT and pN stage but less strongly than for the grade. The association of Ki67 LI with pT stage was primarily driven by low Ki67 LI in pT1 tumors. The association of high Ki67 LI with advanced pN stage was caused by a particularly high Ki67 LI in pN2 stages. A high Ki67 LI was strongly linked to poor patient prognosis (Fig. 3, $p < 0.0001$).

Relationship with molecular features

The Ki67 LI was significantly associated with all molecular features analyzed in this project (Tables IV and V). The Ki67 LI increased significantly with high expression levels of p53 ($p < 0.0001$), p16 ($p < 0.0001$) and Egfr ($p < 0.0001$), as well as in

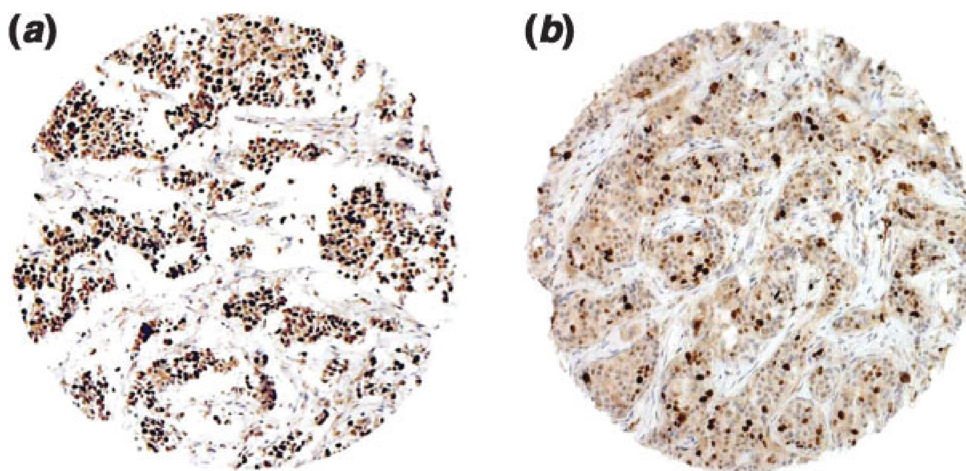

Figure 1 – Representative images of tumors with Ki67 LI. (*a*) Image of a TMA spot with high Ki67 LI. (*b*) Image of a TMA spot with low Ki67 LI.

TABLE III – Ki67 LI VS. CLINICOPATHOLOGICAL PARAMETERS

Category	*N*	Ki67 LI (SD)	95% CI	*p* value[1]
BRE grade				
1	442	18.29 (0.49)	17.32–19.27	*<0.0001*
2	744	23.73 (0.44)	22.86–24.60	
3	579	37.88 (0.63)	36.63–39.12	
PT category				
1	611	23.18 (0.55)	22.10–24.26	*<0.0001*
2	831	29.27 (0.54)	28.21–30.33	
3	108	29.78 (1.67)	26.50–33.10	
4	207	27.74 (0.96)	25.84–29.63	
PN category				
0	741	25.97 (0.56)	24.86–27.07	*0.0018*
1	640	27.55 (0.60)	26.36–28.73	
2	103	31.34 (1.39)	28.58–34.10	

[1]*p* value was calculated with ANOVA.

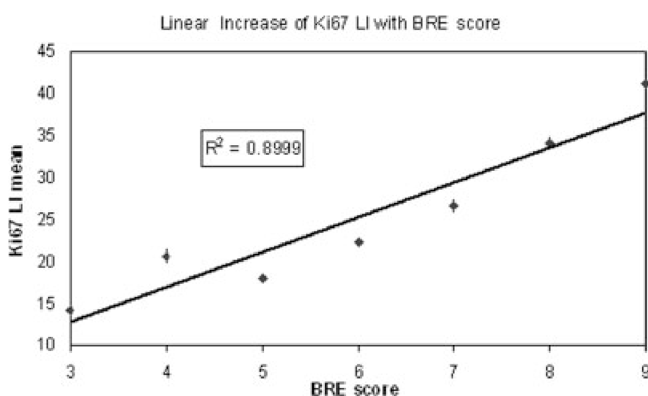

Figure 2 – Ki67 LI *vs*. BRE score. Almost linear increase of Ki67 LI with increase in BRE score.

cases with amplifications of HER2 ($p < 0.0001$), MDM2 ($p < 0.0001$), CCND1 ($p < 0.0001$) or MYC ($p < 0.0001$). The Ki67 LI was found to be significantly decreased in cancers with high expression levels of ER, PR or bcl2 ($p < 0.0001$ each).

Discussion

The results of this study show that sufficiently large TMAs containing just one 0.6-mm sample per tumor are highly efficient to detect associations between molecular features and the Ki67 LI in breast cancer.

The strong associations with tumor phenotype and prognosis found in this study are in line with previous data. The existence of a

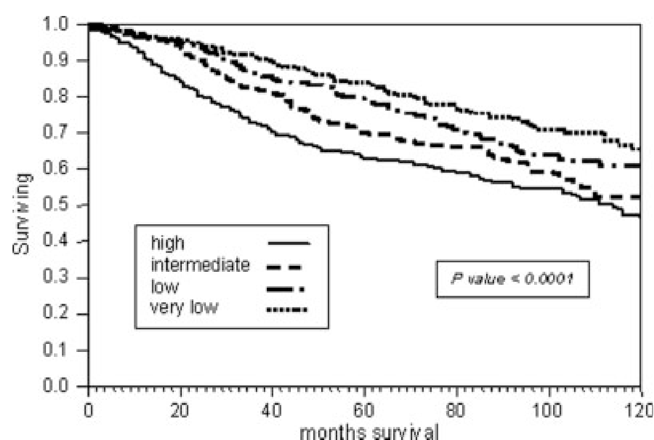

Figure 3 – Ki67 LI *vs*. patient prognosis. High Ki67 LI is strongly linked to poor patient prognosis.

significant association of the Ki67 LI with high grade, advanced stage and poor prognosis of breast cancer patients is largely undisputed as the majority of previous studies, especially the large ones, had overwhelmingly suggested such a relationship.[16–19] Only a minority of studies had failed to find an association with patient survival. These results were most likely due to small patient numbers such as 96,[20] 97[21] or 184[22] in studies showing negative results.

The strong associations found with all individual components of the BRE grade and the almost linear relationship with the BRE score emphasizes the validity of our Ki67 data for research purposes. It is therefore not surprising that highly significant associations were also found with all molecular features analyzed in this study. Importantly, these associations do also include inverse relationships such as with ER and PR expression. This rules out false associations due to reduced immunogenicity in a fraction of samples. In such a situation nonimmunogenic samples would show low or negative results for all antibodies, while well reactive samples had, on average, a higher score. As this would apply for all or most molecular markers, the false statistical associations could be expected.

Our comparison between Ki67 LI and molecular features are well in line with previous data. Associations of Ki67 LI have previously been described with p53,[23] HER2[24] and Egfr,[25] and inverse correlations had been found with ER,[16] PR[23] and bcl2.[26] Again, these findings have sometimes not been confirmed in some small-scale large section studies. For example, some authors[27,28] found no association between ER positivity and Ki67 in a series of 60 and 136 carcinomas, respectively, and Gasparini *et al*.[29] found no association with PR expression in 147 tumors.

TABLE IV – Ki67 LI VS. MOLECULAR FEATURES I

Gene	Ki67 LI				
	Negative	Weak	Moderate	Strong	p value[1]
p53	23.29 (0.43)[2]	26.55 (0.77)	30.34 (1.17)	37.54 (1.06)	*<0.0001*
p16	22.55 (0.53)	23.92 (0.58)	26.46 (0.77)	38.06 (1.06)	*<0.0001*
Egfr	26.20 (0.37)	42.07 (2.69)	40.95 (1.73)	40.86 (1.73)	*<0.0001*
Bcl2	35.01 (1.08)	34.38 (1.36)	27.40 (1.02)	23.90 (0.41)	*<0.0001*

[1]p value was calculated with ANOVA.–[2]Numbers in parenthesis correspond to the SD of the mean.

TABLE V – Ki67 LI VS. MOLECULAR FEATURES II

Gene	Ki67 LI		
	Negative	Positive	p value[1]
HER2 (FISH)	25.16 (0.41)	35.52 (0.94)	*<0.0001*
MYC (FISH)	26.96 (0.41)	39.04 (1.69)	*<0.0001*
CCND1 (FISH)	26.16 (0.41)	31.26 (0.85)	*<0.0001*
MDM2 (FISH)	26.23 (0.38)	33.30 (0.15)	*<0.0001*
ER (IHC)	36.75 (0.84)	24.39 (0.36)	*<0.0001*
PR (IHC)	28.70 (0.48)	24.97 (0.54)	*<0.0001*

[1]p value was calculated with ANOVA.

The number of samples per tumor that should optimally be analyzed on a TMA is disputed in the literature. Existing suggestions are based on 2 types of studies, one comparing TMA data with results obtained on corresponding large sections,[30–39] the other ones comparing TMA data with clinicopathological or molecular features.[6,7,40,41] The first type of studies have generally shown that a good concordance between large section and TMA results exists if only 1 spot per tumor is analyzed and that the concordance further increases with the increase in the number of samples analyzed, and on this basis, some authors have suggested the general use of multiple cores per tumor on the TMA.

Virtually all studies comparing TMA data with clinical or molecular features have only analyzed 1 sample per tumor.[6,7,42–44] Remarkably all these studies found all previously established associations between molecular parameters and other features. As the purpose of TMA studies is to identify clinicopathological associations and not reproducing results previously established on large sections, inclusion of multiple cores per sample may not be advantageous in many instances. The results obtained from 1 TMA spot may be better than the results from large sections, and this was especially demonstrated in one study by Torhorst *et al.*[7] Here, the use of large sections for p53 analysis had not resulted in significant associations with breast cancer prognosis, while a strong correlation with survival was found if one 0.6-mm sample was analyzed on a TMA. Obviously, some of the positive stainings found on large sections were either caused by artifacts or by biologically irrelevant subpopulations.[7] The fact that these tumors that were found to be p53 positive on large sections but not on TMAs had a similarly good prognosis, as entirely p53 negative cases demonstrates that it may not be desirable to reproduce every positive large section finding on a TMA.[45]

Because of its heterogeneous nature, the Ki67 LI was often suspected as a tumor feature that would not be suitable for TMA studies. The results of this study, however, show that Ki67 LI can be excellently analyzed on breast cancer TMAs for research purposes. All 15 expected associations with molecular and clinicopathological features could be found in our TMA study with highly significant p values. A similar result was previously obtained in a bladder cancer TMA containing more than 2,000 samples.[6] Overall these data provide very strong support for the utility of TMAs as a research tool. At least if large enough TMAs are utilized, associations can easily be detected by using the cost efficient 1 core per tumor approach, even for "heterogeneous" markers such as Ki67 LI.

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