

# Limitations of tissue microarrays in the evaluation of focal alterations of bcl-2 and p53 in whole mount derived prostate tissues

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**Abstract.** Several investigators have reported the correlation of p53 and bcl-2 immunoreactivity with post operative prostate specific antigen (PSA) recurrence. Focal and or clustered expression is typical for these biomarkers. The purpose of this study was to compare the effectiveness of tissue microarrays to detect p53 and bcl-2 overexpression and their prognostic significance. Tissue microarrays (TMA) of 99 patients with mean follow-up of 61 months contained 760 samples from 241 carcinomas, 431 benign glands, and 88 foci of prostatic intraepithelial neoplasia (PIN). Overexpression of p53 was seen in 43.3% of 97 patients, whereas bcl-2 overexpression was noted in 23.7% of 97 patients using TMA technology, compared to 66.0% and 26.9%, respectively in the corresponding radical prostatectomy samples. The tissue microarray technology is a powerful tool to study the multifocal and heterogeneous nature of prostate cancer. However, the prognostic value of p53 and bcl-2 could not be confirmed using this technology in contrast to radical prostatectomy sections. The TMA technique is probably more informative and reliable in evaluating the prognostic value of homogeneously expressed biomarkers.

## Introduction

The recently developed tissue microarray technology by Kononen *et al* has the potential to greatly facilitate analysis of alterations in multiple tumor types and histologically heterogeneous areas (1,2). In this technique, 0.6 mm diameter tumor biopsy cores are retrieved from selected regions of archival tissue blocks, and hundreds of such cylindrical samples are subsequently precisely arrayed in a new paraffin block. This provides a high throughput analysis method of multiple targets at the DNA, RNA or protein level (3,4).

Several groups studying the predictive value of p53 and bcl-2 biomarkers in prostate carcinoma have shown that overexpression of p53 and bcl-2 in total prostatectomy specimens correlates with prostate cancer recurrence despite the focal and clustered distribution of these proteins (5-7). Similarly, Kuczyk *et al* found p53 overexpression as a prognostic parameter for recurrence-free survival (8). Our group (5,9) recently evaluated diagnostic needle biopsies for p53 and bcl-2 and could not correlate the frequency of immunohistochemical positivity with our prior corresponding prostatectomy results. Moreover, we did not find any prognostic significance in the needle biopsies most likely due to the sampling related differences between the two techniques (9). The purpose of this study was to investigate tissue microarrays for p53 and bcl-2 protein expression and their prognostic significance in the context of multifocal nature and intratumoral heterogeneity of prostate cancer.

## Materials and methods

**Patient selection and clinical features.** Between 1993 and 1997, 402 radical prostatectomies were performed at the Urology Service of Walter Reed Army Medical Center. Ninety-nine of these patients were selected based on sufficient follow-up, ethnicity and pathological stage. Forty-one had recurrence and 58 were without evidence of

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disease. Eight patients (8.1%) underwent three months of neoadjuvant LH-RH and antiandrogen treatment. The characteristics of the study population are listed in Table I. A subgroup of 12 of these patients was randomly selected for whole mount staining of these biomarkers. Follow-up data were obtained in a prospective manner using the Center for Prostate Disease Research (CPDR) patient database (10). Patients who did not return to our institution for follow-up were contacted and patient information was obtained. Biochemical recurrence was defined as a serum PSA level of 0.4 ng/ml or higher, or two serial measurements of 0.2 ng/ml or greater. Date of recurrence was defined as the earliest date of biochemical or clinical recurrence. Last date of follow-up in patients without recurrence was defined as the date of the most recent serum PSA measurement, or as the date of death in those patients who died without evidence of recurrence.

**Pathologic data.** The total prostatectomy specimens were fixed in 10% buffered formalin, sliced at 2.25 mm intervals, processed, embedded, and sectioned as whole mounts. H&E-stained whole mounted slides from each patient were reviewed by one pathologist (I.A. Sesterhenn) and the slides including the index tumor (largest and worst grade), the highest number of tumors, and the corresponding donor block with at least 1 mm of remaining tissue were selected. Tissues representing tumor regions, benign glands, and prostatic intraepithelial neoplasia (PIN) were marked for tissue array core retrieval (Fig. 1). Each tumor was recorded and graded separately according to the WHO and Gleason score and stage according to the TNM system. Tumors in surgical margins due to capsular invasion were coded as pTx (11-13).

**Prostate tissue array construction.** The prostate TMA was constructed as described by Kononen *et al* (1). With the use of a tissue arraying instrument (Beecher Instruments, Silver Spring, MD) the TMAs were created by punching holes in a 'fresh' recipient paraffin block and retrieving tissue cores from the donor block with a thin-walled needle with an inner diameter of 0.6 mm, held in an x-y precision guide. The cylindrical samples were retrieved from the selected region in the donor block and extruded directly into the recipient block with defined array coordinates. Because the donor blocks were pre-cut and the average sample thickness was only 1 mm, we limited the total samples in the recipient block to 120. To assure adequate representation, two cores were taken from each sample site. This resulted in a total of 760 tissue samples consisting of 241 separate primary tumors, 431 samples of benign glands, and 88 foci of PIN which were arrayed in 10 new paraffin blocks. Fifty-two serial 4-micron sections were taken from each block. The first and last samples were stained with H&E to verify each tissue core. The pathological diagnosis of the tissue cores was saved in the CPDR database for future studies of this TMA (Fig. 1).

**Immunohistochemistry.** The sections were deparaffinized, dehydrated and immersed in 0.6% hydrogen peroxide in methanol to block endogenous peroxidase activity. Antigen retrieval was accomplished by microwaving the sections for 15 min in 10 mM citrate buffer. Immunohistochemical

Table I. Characteristics of 99 radical prostatectomy patients used for tissue microarray analysis.

Variable	No. of pts.	No. of recurrence (%)
Total patients	99	41 (41.4)
Age (years)		
<55	12	7 (58.3)
55-59	24	6 (25.0)
60-64	35	14 (40.0)
65-69	19	9 (47.4)
≥70	9	5 (55.6)
Race		
W	59	14 (35.0)
B	40	27 (45.8)
Stage (pathological)		
pT2b	3	1 (33.3)
pT2c	44	5 (11.4)
pT3a	21	8 (38.1)
pT3b	6	5 (83.3)
pT3c	19	17 (89.5)
pTx	4	3 (75.0)
pT4a	2	2 (100.0)
Stage (grouped) <sup>a</sup>		
PT2	47	6 (12.8)
PT3/4	48	32 (66.7)
Surgical margin		
Positive	40	28 (70.0)
Negative	58	14 (24.1)
Gleason score <sup>b</sup>		
2-4	0	0 (0.0)
5-6	21	4 (19.0)
7	26	14 (53.9)
8-10	15	11 (73.3)
Nuclear grade <sup>c</sup>		
I	32	11 (34.4)
II	51	28 (54.9)
III	10	1 (10.0)
WHO		
Well	14	3 (21.4)
Moderate	32	7 (21.9)
Poor	47	30 (63.9)
Hormonal neoadjuvant therapy		
Yes	8	2 (25.0)
No	91	41 (45.1)

<sup>a</sup>Excluding stage pTx specimen; <sup>b</sup>Unknown for variable, Gleason score- 37; <sup>c</sup>Too little tumor to grade - 6.

detection using commercially available monoclonal antibody for p53 (NCL-1801, Novocastra Labs) and bcl-2 (Dako) was performed. The optimal dilution used for the monoclonal

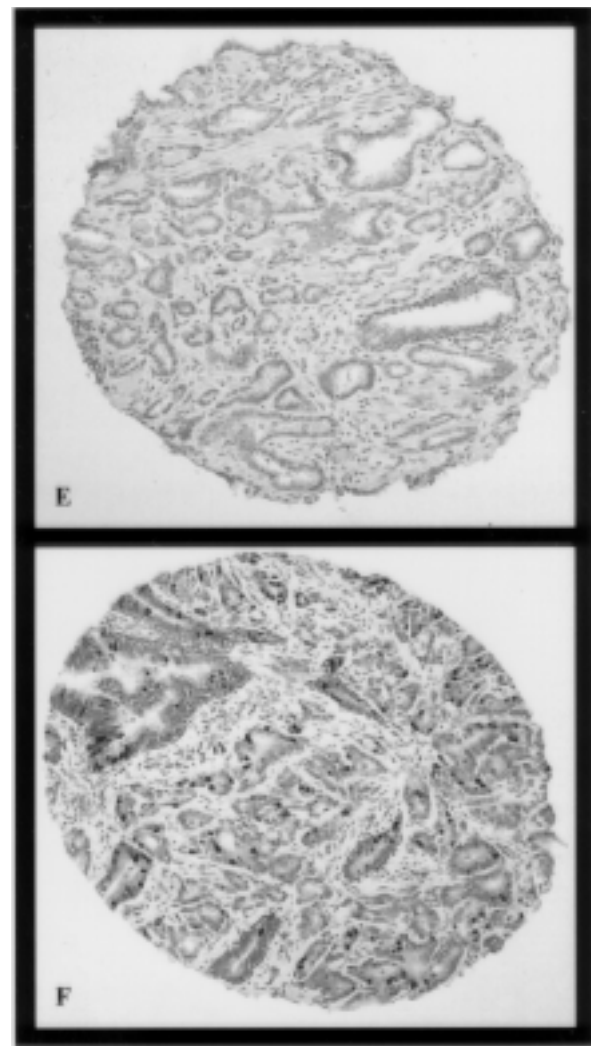
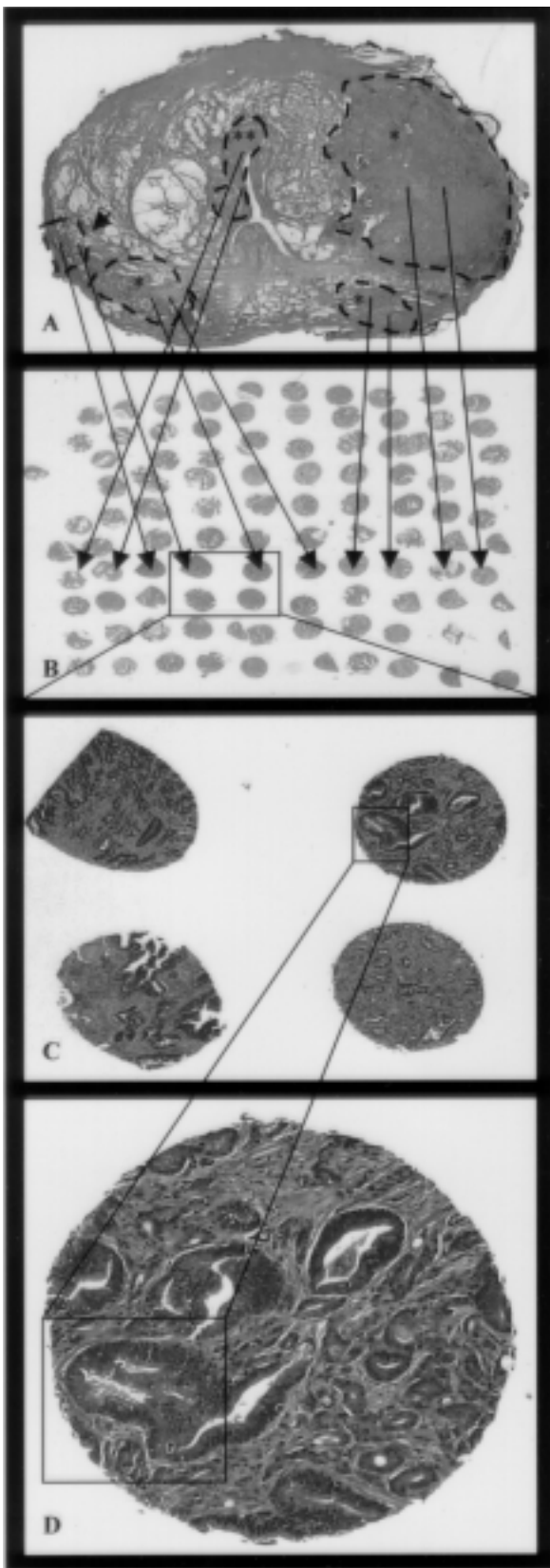


Figure 1. Prostate tissue microarray strategy. A, Close step-sectioned (2.25 mm) radical prostatectomy whole-mounted (H&E) specimen with marked areas for tissue retrieval. Areas of benign glands (double asterisks), carcinoma and prostatic intraepithelial neoplasia (arrowhead) and tumor regions (asterisks). Arrows demonstrating double biopsy retrieval from the same area. B, Tissue microarray sample C, Close up view of four tissue cores (diameter of core 0.6 mm). D, Tissue core containing PIN and carcinoma areas. E, Negative p53 immunohistochemical stain of a tissue core. F, High level (4+) expression of p53 in prostate carcinoma (core diameter 0.6 mm).

antibodies was 1:40 for p53 and 1:160 for bcl-2. The avidin-biotin-peroxidase system (Vectastain Elite Kit; Vector Labs) was used for visualization. Colon cancer samples were used for positive controls for p53 staining, and lymph node samples were used as positive controls for bcl-2. The slides were then

examined and graded for biomarker expression by a single pathologist (I.A. Sesterhenn) as previously described by our group. The cores of each tumor were separately recorded. The patient was given a staining score based on the highest staining of any of the tumors. The pathologist and the

primary investigator (A.S. Merseburger) were blinded to follow-up data during assessment of p53 and bcl-2 staining scores. Positive staining for each case was used to classify the tumor by categories of ‘None’, ‘Rare’ (1-5%), 1+ (6-25%), 2+ (26-50%), 3+ (51-75%) or 4+ (76-100%) of malignant nuclei expressing p53 and/or bcl-2 protein, respectively. The IHC results were calculated counting ‘rare’ pattern IHC staining as negative. The immunohistochemical results of the TMAs were compared with previous results obtained in conventionally processed radical prostatectomies (5) and their corresponding needle biopsies (9).

*Statistical analysis.* Differences in biochemical recurrence-free survival rates were evaluated using the log-rank test for Kaplan-Meier analysis. The results were calculated using ‘rare’ IHC as negative. The histopathologic and clinical data were linked and stored in the CPDR database.

**Results**

Immunohistochemical results for p53 and bcl-2 were evaluated in 97 prostate cancer patients using the tumor tissue cores from radical prostatectomy specimens. Forty-one of the 97 patients (41.4%) with arrayed specimens experienced biochemical recurrence. Eight of the patients underwent hormonal neoadjuvant therapy for 3 months prior to surgery of which three showed positive staining for p53 (37.5%) and five for bcl-2 (62.5%), respectively. Follow-up (in months) ranged from 17 to 89 with a mean value of 61 months. Staining pattern is demonstrated in Table II.

Bcl-2 expression was detected in 23 (23.7%) of the 97 patients. In most cases, the staining was greater than 1+. There was no correlation between the amount of immunohistochemical staining score and recurrence (Table II).

Overexpression of p53 was observed in 42 of the 97 patients (43.3%). Eighteen of these patients showed recurrence (42.9%). In 55 cases without p53 IHC staining, recurrence occurred in 23 (41.8%) (Table II). Fifteen patients stained positive for p53 in one tumor and negative in another within the same radical prostatectomy specimen. In eight patients, two tumors in the same prostate were positive (Fig. 1). The immunostaining of the tissue microarrayed specimens for p53

Table II. Bcl-2 and p53 immunohistochemical staining versus biochemical recurrence in 97 radical prostatectomy microarrayed patients.<sup>a</sup>

IHC score	Total no. of patients	No. of recurrence (%)	p-value <sup>a</sup>	
<b>bcl-2</b>				
Negative	72	29 (40.3)	p=0.257	
Rare	2	1 (50.0)		
1+	5	2 (40.0)		
2+	6	4 (66.7)		
3+	7	4 (57.1)		
4+	5	1 (20.0)		
Positive	23	11 (47.8)		
Negative	74	30 (40.5)		
<b>p53</b>				
Negative	38	14 (36.8)		p=0.576
Rare	17	9 (52.9)		
1+	29	17 (58.6)		
2+	1	0 (0.0)		
3+	4	0 (0.0)		
4+	8	1 (12.5)		
Positive	42	18 (42.9)		
Negative	55	23 (41.8)		
<b>p53 + bcl-2</b>				
Both positive	12	5 (41.7)	p=0.231	
Both negative	32	12 (37.5)		

<sup>a</sup>Using log-rank test.

or for bcl-2 was not predictive of recurrence. Kaplan-Meier curves for both proteins in the TMAs did not discriminate between patients with or without recurrence (p=0.257, p=0.576, respectively as shown in Fig. 2).

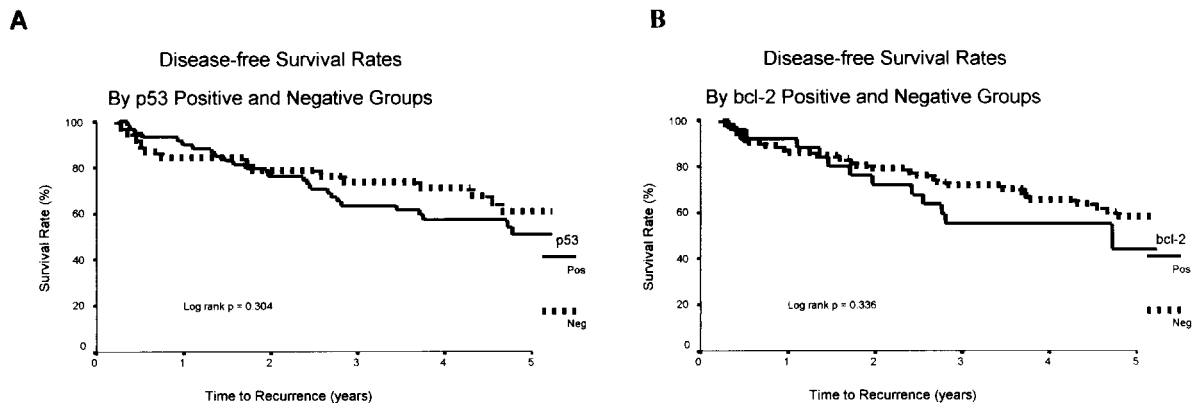


Figure 2. Kaplan-Meier disease-free survival curves for tissue microarrays of (A) p53 and (B) bcl-2.

## Discussion

The significance of p53 and bcl-2 mutations in primary prostate cancer remains controversial. However, there appears to be some consensus on higher frequency of p53 alterations in advanced stage disease. Similarly, bcl-2, in general, is overexpressed in more advanced disease.

The p53 tumor suppressor gene, the most frequently altered gene in human cancers, is a cellular gatekeeper and protects the genome against genotoxic and other types of stress (reviewed in ref. 14). The p53 gene resides on chromosome 17p13 and deletions of the wild-type (wt) p53 allele in this region are common in a wide variety of human tumors (15). The remaining p53 allele in those tumors frequently harbors point mutations. In particular, exons 5 through 8 have been noted to be 'hot spots' for gene mutations in many human cancers. The normal or wt p53 protein has a very short half-life, while a significant proportion of altered/ mutated proteins has much longer half-life. This property of the mutant p53 protein allows it to be more easily detected by IHC methods which indirectly suggests for p53 gene alteration (16).

A number of studies using different analytical tools to include IHC, single strand conformation polymorphism (SSCP), and DNA sequencing, have yielded widely varying rates of p53 mutation including overexpression in human prostate cancer specimens. Positive immunostaining ranged from only 4% (17), to 79% (18) of untreated prostate cancers. The discrepancy in the IHC detection of p53 among different groups could be due to differences in the definition of positivity, the selection and dilution of antibodies, and fixation differences. Another possibility is the selection of the tumors to be evaluated. Quinn *et al* have reported the largest study to date with 263 radical prostatectomy patients followed for a mean of 55 months with a positivity of 50.2% (19). p53 nuclear accumulation, detected by IHC of radical prostatectomy specimens, as determined by a percentage of positive malignant cells which is usually clustered and very focal, was a strong independent predictor of recurrence after surgery. Through careful analysis, Mirchandani *et al* showed that within radical prostatectomy specimens there was widespread heterogeneity of p53 mutations from tumor to tumor and within single tumors. They proposed that this might be responsible, at least in part, for the wide variations reported in mutation rates (7,20). By laser capture microdissection of p53 immunostained regions of the prostate, Griewe *et al* have confirmed mutations by DNA analysis even in regions with rare tumor cells positive for p53 (unpublished data). This study also emphasizes that refined techniques increase the yield of detecting p53 alterations.

The proto-oncogene bcl-2 encodes a protein product, which functions as an inhibitor of apoptosis (21). Cancer associated bcl-2 overexpression has been implicated in tumorigenesis (22). Bcl-2 has been less thoroughly studied in prostate cancer than p53. Overexpression by IHC is seen in a lower percentage of primary prostate tumors than p53 expression. Bauer *et al* from our laboratory showed that bcl-2 expression in the radical prostatectomy specimens was a significant predictor of biochemical (PSA) recurrence (5). Keshgegian *et al* studied 208 consecutive radical prostatectomy specimens finding that bcl-2 and Ki-67 (MIB-1) IHC

were independent predictors of recurrence (23). However, the value of bcl-2 even in radical specimens is controversial. Johnson *et al* (24) found that bcl-2 expression was infrequent and not prognostic, but Bylund *et al* (25) found that bcl-2 overexpression was associated with better outcome.

Bauer *et al* have shown that immunohistochemical staining of radical prostatectomy specimens for the protein products of bcl-2 and p53 can be of prognostic value, despite their very focality and clustered expression (5). Stackhouse *et al*, also from our group, compared the results of p53 and bcl-2 overexpression in needle biopsies of the same patient group with progression. In this group, p53 and bcl-2 detection was of no prognostic significance. As one would expect, in the TMAs the results are similar to the needle biopsies, even by taking two samples from each tumor.

The contemporary whole-mounted radical prostatectomies from our institution typically reveal about three separate tumors per prostate (26). Byar and Mostofi showed that prostate carcinomas were multifocal and that each tumor had a heterogeneous appearance in the H&E stained sections (20). They postulated that large tumors are probably the result of merging smaller tumors. More recently, Miller and his co-workers have shown similar results (27). Due to this multifocality and heterogeneity sampling differences in designing TMAs of prostate carcinomas may occur, and may account for our finding that p53 and bcl-2 staining of the TMAs does not provide the same prognostic information which was found upon staining of the prostatectomy specimen. Mucci *et al* support the use of TMAs, even in focally expressed biomarkers in advanced prostate cancer by increasing the number of tissue samples taken, but limit its use for screening of new antibodies. They comment: 'In a clinical setting where prognosis is based on a focal event, standard slides would be more appropriate' (28). Our study supports the finding that focally expressed biomarkers fail to show prognostic significance in TMAs similar to needle biopsies. Even with the knowledge of the location of the tumor, we could not increase the yield of positive findings compared to biopsy findings (9). It is conceivable that additional samples from each tumor may have resulted in a greater number of positive cases. Although we had relatively long follow-up (mean 61 months), the number of cases may have been insufficient to ascertain subtle prognostic value of the biomarkers being studied. Furthermore, the fact that eight cases received neo-adjuvant therapy could have affected the results.

While it is possible to use the TMA technique even in thin donor blocks, technical problems in cutting the recipient blocks limits the number of tissue cores in this block.

As described by various groups, there is no doubt that the TMA technology has a great potential to significantly accelerate molecular studies that seek associations between molecular changes and clinicopathologic features of cancer (2,29-31). However, the value of TMAs to find prognostic markers may be limited to biomarkers that are widespread within the tissue and not focally clustered like the ones evaluated in this study.

In conclusion, we have established a strategy to develop TMAs from multisampled whole mounted radical prostatectomy sections. In our group of 241 prostatic carcinomas, the heterogeneity, focality, and clustered

expression of p53 and bcl-2 are expected problems in the use of TMAs. Apparent discrepancies in p53 and bcl-2 results among the four types of specimens (conventional prostatectomy, biopsy and TMAs) underscore the importance of sampling issues associated with biomarker analysis in clinical specimens. We conclude that the use of biomarkers, which are more uniformly expressed in prostate cancer, should be the choice if TMAs are used to detect their prognostic significance.

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