Diagnostic and prognostic value of T-cell receptor gamma alternative reading frame protein (TARP) expression in prostate cancer

Florian R. Fritzsche[1]*, Carsten Stephan[2]*, Josefine Gerhardt[1],
Michael Lein[2,3], Irina Hofmann[1], Klaus Jung[2,3], Manfred Dietel[4] and Glen Kristiansen[1]

[1]Department of Surgical Pathology, University Hospital Zurich, Zurich, Switzerland, [2]Department of Urology, Charité – University Hospital, Berlin, Germany, [3]Berlin Institute for Urologic Research, Berlin, Germany and [4]Institute of Pathology, Charité – University Hospital, Berlin, Germany

* both authors contributed equally to this work

Summary. T-cell receptor gamma chain alternative reading frame protein (TARP) has recently been proposed as being up-regulated in prostate cancer (PCA). Additionally, TARP has been proposed as a potential therapeutic target for cancer therapy. We analysed the protein expression of TARP in a large well characterised prostate cancer cohort to assess its diagnostic and prognostic value. Methodologically, we constructed a tissue microarray comprising more than 600 PCA cases including matching benign prostate tissue. TARP protein expression was carefully analysed and associated with clinico-pathological parameters, PSA-relapse free survival and expression data of established and proposed diagnostic markers (AMACR, p63, GOLPH2). Our results show that TARP is significantly over-expressed in the vast majority (~85%) of PCA in comparison to non neoplastic prostate tissue. Its expression was associated with conventional markers of unfavourable and more aggressive tumour behaviour. However, a prognostic value of TARP could not be found. The diagnostic value of TARP is limited in comparison to AMACR, p63 or GOLPH2. Since TARP specific immunologic therapy regimen are currently being tested, the high frequency of TARP over-expression in PCA conveys a high potential for a predictive and potentially therapeutic use of this biomarker.

Offprint requests to: Glen Kristiansen, MD, Institute for Surgical Pathology, Department of Pathology, University Hospital Zurich (USZ), Schmelzbergstr. 12, 8091 Zurich, Switzerland, e-mail: glen.kristiansen@usz.ch

Key words: TARP, Immunohistochemistry, Diagnostic marker, Prostate cancer, Prognosis

Introduction

Prostate cancer is one of the most common types of cancer and a relevant cause of cancer associated deaths in the United States of America and worldwide (Coleman et al., 2008; Jemal et al., 2008). Prostate cancer relapse after initial surgical/radiotherapeutic treatment and following hormone ablative therapy is often associated with cancer related death due to missing treatment options in this situation. Therefore, there is still an urgent need to find sensitive and specific tissue- and serum-based biomarkers for diagnostic, predictive and prognostic purposes to achieve a better risk- and therapy stratification/individualisation to reduce the mortality of prostate cancer (Parekh et al., 2007). Expression arrays, SNP analyses and mass spectrometry are novel tools for biomarker identification (Zheng et al., 2007). Alpha-metyl-Co-racemase, AMACR, is one example of proteins being detected that way. First described up-regulated in prostate cancer by Xu et al. AMACR has become a clinically accepted and widely used diagnostic marker in prostate cancer (Xu et al., 2000; Rubin et al., 2002). AMACR is frequently used in combination with the basal cell markers p63, CK5/6 and 34betaE12 since the complete absence of basal cells in glandular proliferations in the prostate is a defining criterion of invasive prostate cancer (Brawer et al., 1985). Especially in needle biopsies with sometimes minute amounts of suspicious glands, additional

diagnostic biomarkers might prove important. AMACR can be helpful in such cases, but false negative rates of up to 18% have been reported (Epstein, 2004; Zhou et al., 2004).

Recently we and others have identified GOLPH2 (Golgi phosphoprotein 2) as a tissue-biomarker of prostate cancer (Kristiansen et al., 2008; Varambally et al., 2008; Wei et al., 2008).

Another candidate marker that has been identified in several array based transcript profiling studies of prostate cancer is T-cell receptor gamma alternative reading frame protein (TARP) (Kristiansen et al., 2005; Schlomm et al., 2005). In this study we analysed the protein expression of TARP, which is considered a breast and prostate cancer specific antigen, in a well characterised large prostate cancer cohort with matched normal prostate tissue in direct comparison to AMACR and p63 to evaluate its diagnostic or even prognostic value.

Materials and methods

Prostate cancer patients

Six-hundred-twenty-one prostate cancer patients who underwent radical prostatectomy between 1999 and 2005 were enclosed in this study. Patient age ranged between 43 and 74 years (median 62). Pre-operative Prostate specific antigen (PSA) levels ranged from 0.8 to 39 ng/ml (median 7.2). Forty-four patients (7.1%) had received gonadotropin-releasing hormone analogues at the discretion of the referring urologist prior to surgery (median 4 weeks, range 2-16 weeks). Clinical follow-up data were annually assessed. PSA relapse free survival time was available for 582 patients. The median follow-up time of all cases was 47.5 months (range 1-94 months). 85 patients (14.6%) experienced a PSA relapse. The Gleason scores (GS) in the cohort were distributed as follows: GS 2-6: 220 (35.4%) GS 7: 292 (47.0%), GS 8-10: 109 (17.6%). Four-hundred-twenty-six cases had organ confined carcinomas (pT2), 194 cases showed extracapsular tumour extension (pT3). The surgical margins were clear (R0) in 450 cases, 168 cases had positive margins (R1), 3 cases were Rx. Use of this tissue has been approved by the Charité University Ethics Committee under the title 'Retrospektive Untersuchung von Gewebeproben mittels immunhisto-chemischer Färbung und molekularbiologischer Methoden' ('Retrospective analysis of tissue samples by immunohistochemistry and molecular biological techniques') (EA1/06/2004) on 20 September 2004.

Cell culture

The cell lines used in this study were purchased from ATCC. The human prostate epithelial cell line RWPE-1 was cultured in Keratinocyte-SFM Medium supplemented with EGF and BPE. The human prostate cancer cell lines LNCaP, DU-145 and PC-3 were maintained in RPMI 1640, Minimum Essential Medium and F-12K Medium, respectively, each supplemented with 10% FBS (all media and supplementaries from life technologies, Carlsbad, CA, USA).

Prostate tissue microarray construction

A tissue microarray was constructed as described before (Kristiansen et al., 2008). Each cancer case was represented by five tissue cores with two cores from the invasive carcinoma, two cores from benign prostatic glands and, if present, one core from prostatic intraepithelial neoplasia (PIN). If no PIN was available, an additional core from non-neoplastic tissue was included. The core diameter was 1.5 mm.

Immunohistochemistry

The TMA blocks were freshly cut (3 μm) and mounted on superfrost slides (Menzel Gläser). Immunohistochemistry for TARP was conducted with the Bond automated staining system (Leica Microsystems, Heerbrugg, Switzerland) using Bond reagents for the entire procedure. Antigen retrieval was conducted according to the machine's H2(60) protocol. To detect TARP we used a monoclonal antibody (clone eBioTP1 (1F8), eBioscience Inc., San Diego, CA, USA) with a dilution of 1:200.

To detect racemase and p63, we created a cocktail of racemase (rabbit polyclonal, Biologo, Kronshagen, Germany, dilution 1:30) and p63 (clone mix 4A4/Y4A3, Neomarkers, dilution 1:200) in a Ventana diluent. GOLPH2 immunohistochemistry was conducted as described before (Kristiansen et al., 2008).

Western blotting

Cells were lysed in 60 mM n-Octyl-ß-D-glucopyranoside in the presence of protease inhibitors. Lysates were cleared by centrifugation at 21000 g for 15 min at 4°C and the supernatants stored at -20°C until analysis. Twenty μg of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12.5% w/v) and transferred onto a polyvinylidene difluoride (PVDF) membrane. TARP protein was detected by an antihuman TARP monoclonal antibody (eBioTP1, eBioscience, San Diego, CA, USA; 0.5 μg/ml). As loading control the blots were reprobed with an antihuman Actin monoclonal antibody (MAB1501, Millipore, Billerica, MA, USA). Detection of the primary antibody was performed using horseradish peroxidase (HRP)-conjugated secondary antibodies and SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology, Rockford, IL, USA).

Evaluation of the immunohistochemical stainings

TARP immunohistochemistry was evaluated by two genitourinary pathologists on a multiheaded microscope. We used a printout of the following four different

intensity levels to ensure a stringent evaluation. For TARP, as well as for GOLPH2 and racemase we evaluated staining intensity with a four-tier system: 0 (negative), 1+ (weak), 2++ (moderate), 3+++ (strong) in benign tissue, PIN and invasive carcinoma.

To detect also very subtle staining intensity differences, we further created a dichotomous ("tumour>normal") ratio to better indicate up-regulation in tumour in comparison to adjacent normal tissue. Equal or less TARP staining intensity in carcinomatous tissue was reported as ratio 0, higher staining intensities than in normal glands were regarded as ratio 1.

Heterogeneity of TARP expression in invasive carcinoma was also recorded and diagnosed if more than 25% of the tumour showed a variation of staining intensity exceeding one scoring category.

Statistical analysis

Statistical analysis was performed using SPSS, version 15.0. P-values <0.05 were considered significant.

Results

TARP Western blots

The Western blots for TARP showed single specific bands in the LNCaP, DU-145 and RWPE-1 cell lines but no TARP signal in the PC-3 cell line. If expressed,

TARP was clearly up-regulated in the carcinoma cell lines in comparison to the non-neoplastic cell line RWPE-1 (Fig. 1).

TARP Immunohistochemistry

TARP was expressed in the cytoplasm of almost all (98.7%) prostate cancers with a median staining intensity of 2 (Fig. 2). TARP was also expressed in 66% of the matched normal prostate tissue with a median
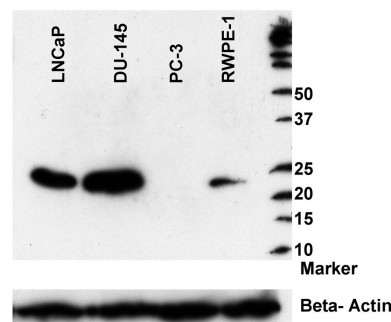
Fig. 1. Western blot for TARP in the prostate cancer cell lines LNCaP, DU-145 and PC-3 compared to the non-neoplastic prostate cell line RWPE-1. The antibody demonstrates distinctive bands for the LNCaP, DU-145 and RWPE-1 cell lines. For PC-3 no TARP signal was detected, while the Actin control was positive. In the cancer cell lines LNCaP and DU-145 TARP was up-regulated in comparison to the RWPE-1 cell line.

Table 1. TARP protein expression in prostate cancer and associations with clinico-pathological parameters.

		TARP Expression					
		All	0	1+	2+	3+	p-value
All cases		621 (100)	8 (1.3)	99 (15.9)	370 (59.6)	144 (32.2)	
Age	≤62	312 (50.2)	4 (1.3)	61 (19.6)	178 (57.1)	69 (22.1)	0.078
	>62	309 (49.8)	4 (1.3)	38 (12.3)	192 (62.1)	75 (24.3)	
Pre-op PSA*	≤10ng/ml	447 (72.0)	6 (1.3)	77 (17.2)	282 (63.1)	82 (18.3)	<0.001
	>10ng/ml	166 (26.7)	2 (1.2)	20 (12.0)	84 (50.6)	60 (36.1)	
pT-status	pT2	426 (68.6)	6 (1.4)	74 (17.4)	264 (62.0)	82 (19.2)	0.002
	pT3/4	195 (31.4)	2 (1.0)	25 (12.8)	106 (54.4)	62 (31.8)	
Gleason sum	3-6	220 (35.4)	1 (0.5)	45 (20.5)	139 (63.2)	35 (15.9)	
	7	292 (47.0)	3 (1.0)	43 (14.7)	171 (58.6)	75 (25.7)	0.006
	8-10	109 (17.6)	4 (3.7)	11 (10.1)	60 (55.0)	34 (31.2)	
Residual status**	R0	450 (72.5)	6 (1.3)	76 (16.9)	271 (60.2)	97 (21.6)	0.121
	R1	168 (27.1)	2 (1.2)	23 (13.7)	97 (57.7)	46 (27.4)	
GOLPH2***	1+	10 (1.6)	1 (10.0)	2 (20.0)	6 (60.0)	1 (10.0)	
	2+	275 (44.3)	3 (1.1)	51 (18.5)	158 (57.5)	63 (22.9)	0.097
	3+	327 (52.7)	4 (1.2)	43 (13.1)	202 (61.8)	78 (23.9)	
AMACR****	0	20 (3.2)	2 (10.0)	12 (60.0)	3 (15.0)	3 (15.0)	
	1+	101 (16.3)	2 (2.0)	19 (18.8)	59 (58.4)	21 (20.8)	<0.001
	2+	325 (52.3)	3 (0.9)	50 (15.4)	203 (62.5)	69 (21.2)	
	3+	173 (27.9)	1 (0.6)	18 (10.4)	103 (59.5)	51 (29.5)	

*: for 8 cases no preoperative (pre-op) PSA value was available, **: 3 cases were Rx, ***: for 9 cases no GOLPH2 value was available, ****: for 2 cases no AMACR value was available.

staining intensity of 1 (Fig. 2). The differences between these two groups were statistically significant (p=0.002, Fig. 3). In 533 cases (85.8%) TARP expression in the tumour was considered higher than in the normal tissue. The comparison with those cases where a matching AMACR (n=619) and GOLPH2 (n=612) staining was available confirmed these data (Table 1). TARP expression in the tumour was considered higher if compared with normal prostate tissue in 85.8%, while the respective percentages for GOLPH2 and AMACR were 92.5% and 94.8%. In 1.8% and 2.4% of cases even the combination TARP/GOLPH2 and TARP/AMACR failed to discriminate the tumour from the benign tissue.

Associations and correlations with clinico-pathological parameters

TARP staining intensity was associated with higher pre-operative PSA levels (p<0.001), advanced pT-status (p=0.002) and higher Gleason score (p=0.006). Age and residual tumour status were not significantly associated with TARP expression. Furthermore, TARP expression in prostate cancer was significantly associated with AMACR expression (p<0.001) and showed a trend for an association with GOLPH2 expression (p=0.097; Table 2).

PSA relapse free survival

While all conventional prognosticators (pre-operative PSA, pT-status, Gleason score and residual status) were highly significant predictors of shortened PSA relapse free survival times (all p<0.001), TARP expression was not (Fig. 4). Although the Kaplan Meier curves showed a slight trend for shortened relapse free times for higher TARP expressing tumours, significance was never reached independency from the cut-off level applied (median, quartiles).
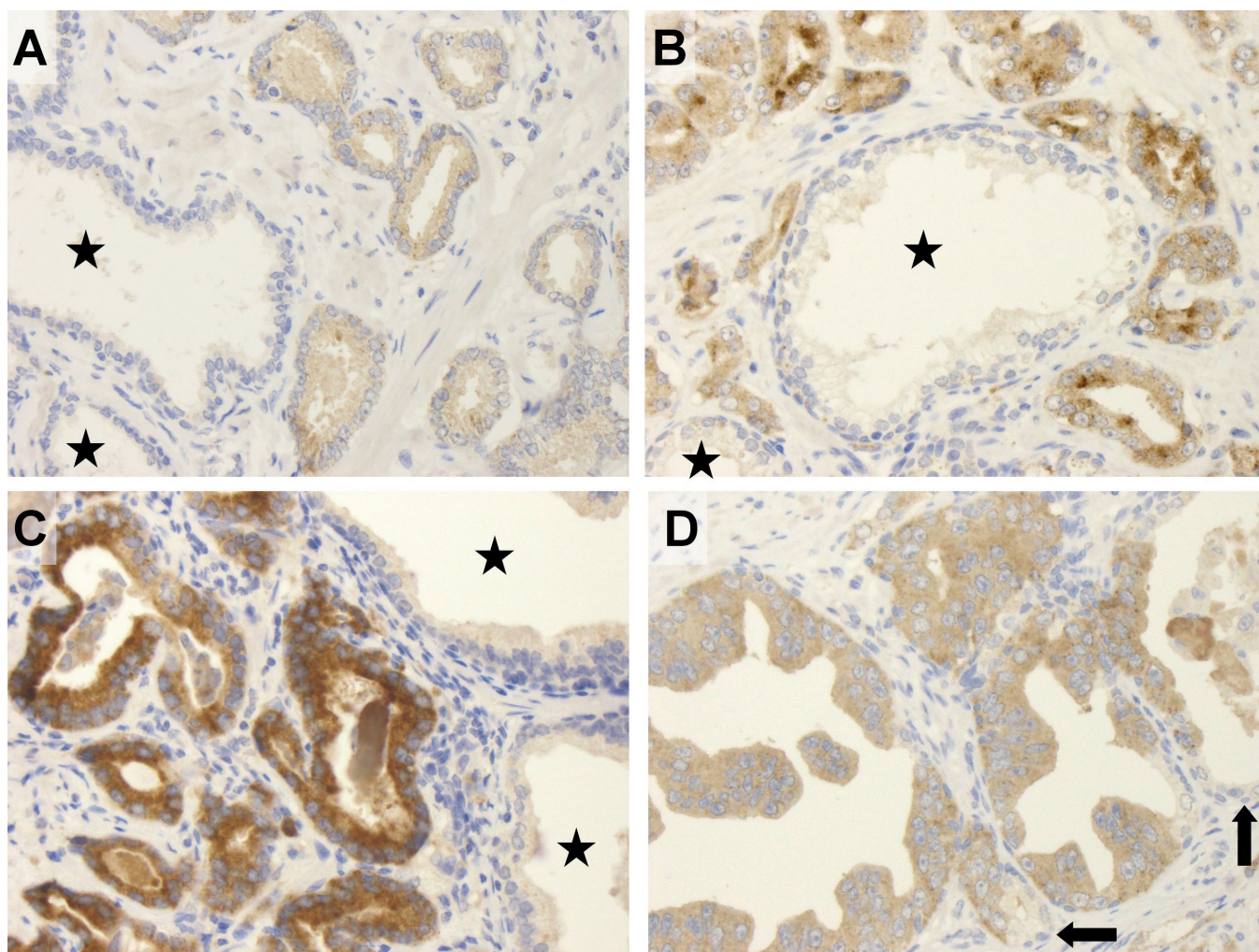

Fig. 2. TARP immunohistochemistry. **A-D**: Prostate cancer with weak **(A)**, moderate **(B)** and strong **(C)** TARP protein expression and adjacent benign prostate glands (star). In PIN the TARP expression was often equal to that in prostate cancer **(D)**. x 200

Table 2. Tumour/Normal ratio of TARP in comparison with AMACR and GOLPH2 expression in prostate cancer.

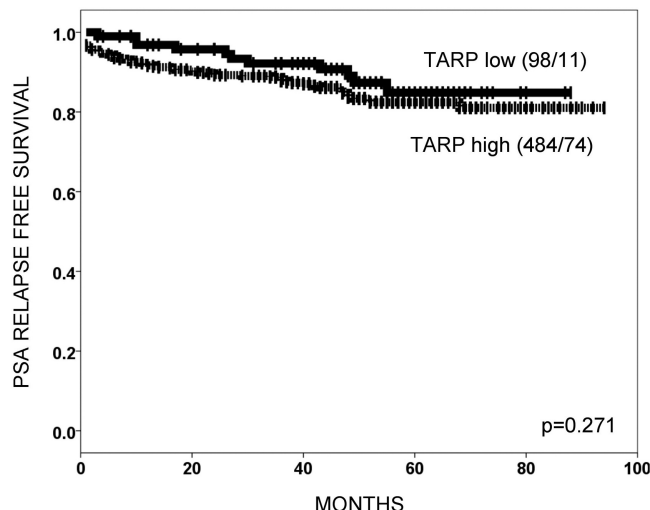|		TARP tumour>normal		Total GOLPH2/AMACR
		No	Yes	
GOLPH2 tumour>normal	No	11	35	46 (7.5)
	Yes	76	490	566 (92.5)
Total TARP		87 (14.2)	525 (85.8)	
AMACR tumour>normal	No	15	17	32 (5.2)
	Yes	73	514	587 (94.8)
Total TARP		88 (14.2)	531 (85.8)	
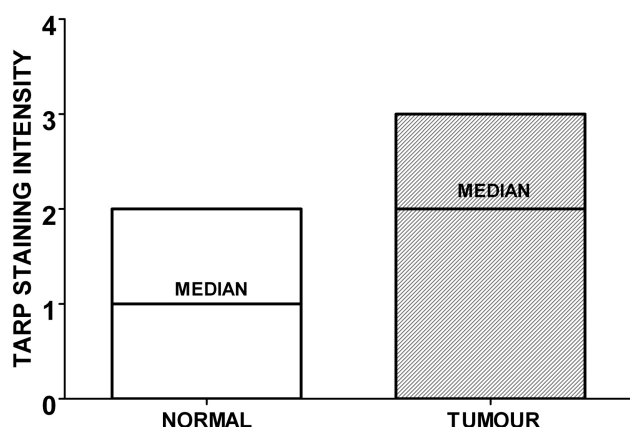
Fig. 3. Bar diagram illustrating TARP expression in normal (white) and tumourous (striated) prostatic tissue. TARP protein expression is significantly higher in prostate cancer (right bar, median 2) compared with normal tissue (median 1).

Fig. 4. PSA-relapse free survival curve for TARP protein expression. Patients with higher TARP expression (bold line) show a tendency for shortened disease free survival in comparison to patients with lower TARP expression (dotted line). This slight trend might be explained by the associations of TARP expression with conventional parameters of tumour progression and aggressiveness. However, the demonstrated trend did not reach significance.

Discussion

This study is the first description of TARP expression as detected by immunohistochemistry in a large clinically well characterised prostate cancer cohort. We were able to confirm the up-regulation of TARP protein in prostate cancer as proposed by Schlomm and Kristiansen et al. on mRNA level (Kristiansen et al., 2005; Schlomm et al., 2005).

In prostate cancer, TARP was first described and named by Wolfgang and Brinkmann and colleagues who detected TARP and its gene in normal prostate glands and the LNCaP prostate cancer cell line (Brinkmann et al., 1998; Wolfgang et al., 2000). Additionally, they demonstrated that TARP over-expression in prostate cancer cells showed increased proliferation rates and that TARP expression could be enhanced by testosterone (Wolfgang et al., 2000, 2001). The latter was also found by Cheng et al. (2003) and clearly makes TARP a possibly important target gene in prostate cancer, which is in its early stages an androgen dependent neoplasm.

Although we found TARP expressed in almost all prostate cancers, the direct comparison to matching and adjacent normal tissue revealed a less impressive rate of up-regulation in 85% of cases. This might seem high, but from the diagnostic standpoint it implies that up to 15% of cases are not adequately labelled and correctly identified as malignant. AMACR is, here, with 95% over-expression, clearly superior. In comparison to AMACR, which can label high grade PIN and sometimes to a low degree also hyperplastic glands of the transitional zone, TARP appears to be up-regulated at fairly early stages, since many glands with low grade PIN features are strongly decorated by TARP. This leads to a weak contrast to invasive glands and clearly limits its use as a complementary diagnostic marker. Nonetheless, in rare cases a combined use of AMACR and TARP might be helpful.

TARP has been discussed as a direct downstream target of the homeobox transcription factor C6 (HOXC6), which is known to be over-expressed in prostate cancer and is thought to play a crucial role in prostate cancer progression by its interactions with the Wnt and Notch pathways (McCabe et al., 2008). This could imply a prognostic value of TARP. Indeed, we found TARP protein expression to be associated with markers of unfavourable patient outcome, although a prognostic value could not be demonstrated. This might, of course, be due to the high rate of overall positivity in prostate cancer, which does not allow delineating meaningful patient subgroups. This compares to the established diagnostic markers for prostate cancer AMACR or GOLPH2 that are not used either to estimate patient prognosis in prostate cancer.

Cheng et al. have proposed a TARP-promoter-based

adenovirus as a potential gene therapy for prostate cancer (Cheng et al., 2004). Subsequently, Carlsson et al. generated T cells against TARP and suggested TARP likewise as a cellular target for immunotherapy (Carlsson et al., 2004). Similar results were published by Kobayashi et al. (2005). Although our data and the high rate of over-expression do in principle support the view of TARP as an appealing candidate for targeted therapy, its intracellular localisation might prove difficult. Pastan et al., who also detected TARP in breast cancer cell lines, localised TARP in the mitochondria of prostate cancer cells (Wolfgang et al., 2000; Maeda et al., 2004), which also explains the slightly granular immunoreactivity we observed. Oh et al. developed epitopes against TARP, and a very recent work of Epel et al. from the same working group has demonstrated that TARP could be targeted by T cell receptor-like human recombinant antibodies (Oh et al., 2004; Epel et al., 2008). With these results and with its demonstrated high expression in prostate cancer, TARP might indeed constitute a potential target for future chemotherapeutic approaches. In this constellation, TARP expression analyses might become helpful as predictive tool in assessing prostate cancer.

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