Pathol. Oncol. Res. (2014) 20:727–732
DOI 10.1007/s12253-014-9753-2

RESEARCH

Transforming Growth Factor-Beta Signaling Leads to uPA/PAI-1 Activation and Metastasis: A Study on Human Breast Cancer Tissues

D. S. Lang · S. Marwitz · U. Heilenkötter · W. Schumm · O. Behrens · R. Simon · M. Reck · E. Vollmer · T. Goldmann

Received: 18 July 2013 / Accepted: 20 February 2014 / Published online: 9 March 2014

Abstract Metastasis represents a major problem in the treatment of patients with advanced primary breast cancer. Both Transforming Growth Factor-Beta (TGF-β) signaling and Plasminogen Activator (PA) components, urokinase-type Plasminogen Activator (uPA) and Plasminogen Activator Inhibitor-1 (PAI-1) represent a complex network crucial for such enhanced invasiveness of tumors and imply high prognostic/predictive and promising therapeutic potential. Therefore, protein expression of specific effector molecules comprising the main parts of the TGF-β signaling pathway were determined in HOPE-fixed human tumor tissues through IHC (Scoring) using tissue microarray (TMA) technique and correlated with respective uPA and PAI-1 levels determined earlier in the same TMAs through optimized IHC and semi-quantitative image analysis. TGF-β signaling was active in vast majority (96 %) of the tumor samples and 88 % of all cases were significantly correlated with established metastasis markers uPA and PAI-1. In addition, TGF-β was also closely associated with tumor size, nodal status and two steroid hormone receptors. Consistent interrelationships between TGF-β, PA components and additional tumor characteristics underline the superiority of such more comprising data with regards to confirming TGF-β signaling as a promising target system to inhibit metastasis in advanced breast cancer.

D.S. Lang and S. Marwitz contributed equally to this research.

D. S. Lang (✉) · S. Marwitz · E. Vollmer · T. Goldmann
Clinical and Experimental Pathology, Research Center Borstel, Parkallee 3a, 23845 Borstel, Germany
e-mail: dlang@fz-borstel.de

S. Marwitz
e-mail: smarwitz@fz-borstel.de

E. Vollmer
e-mail: evollmer@fz-borstel.de

T. Goldmann
e-mail: tgoldmann@fz-borstel.de

D. S. Lang · S. Marwitz · M. Reck · E. Vollmer · T. Goldmann
Airway Research Center North (ARCN)- Member of the German Center for Lung Research (DZL), Borstel, Germany

M. Reck
e-mail: m.reck@lungenclinic.de

U. Heilenkötter
Clinical Center Itzehoe, Gynecological Hospital, Holsteinisches Breast Center, Itzehoe, Germany
e-mail: u.heilenkoetter@kh-itzehoe.de

W. Schumm
imland Clinic Rendsburg, Pathology, Holsteinisches Breast Center, Rendsburg, Germany
e-mail: wolfgang.schumm@dgn.de

O. Behrens
imland Clinic Rendsburg, Gynecological Hospital, Holsteinisches Breast Center, Rendsburg, Germany
e-mail: oliver.behrens@imland.de

R. Simon
University Medical Center Hamburg Eppendorf, Pathology, Hamburg, Germany
e-mail: r.simon@uke.uni-hamburg.de

M. Reck
LungenClinic Grosshansdorf, Oncology, Großhansdorf, Germany

Keywords Metastasis · Breast cancer · Urokinase-type plasminogen activator · Plasminogen activator inhibitor-1 · Transforming growth factor beta · Mothers against decapentaplegic homolog 3 · BMP Activin membrane-bound inhibitor homolog

Introduction

Increasing knowledge about the molecular mechanisms underlying metastasis in breast cancer is crucial to identify new targets in order to develop strategies for anticancer treatment [1, 2]. Invasiveness of breast cancer is related to enhanced extracellular matrix degradation mediated by increased secretion of the two central components of the PA system, uPA and PAI-1, also involved in tumor migration and angiogenesis [3, 4]. In consequence, the combined overexpression of these regulator proteins in tumor tissues was demonstrated to be predictive for risk identification in breast cancer patients [5, 6]. Likewise, activation of the TGF-β pathway was observed in many tumors including breast cancer; promotes progression through direct regulation of uPA or PAI-1, as demonstrated in the MDA-MB-231 cell line or human hepatocellular carcinoma HepG2 cells, respectively [7, 8]. Canonical TGF-β signaling is mediated by phosphorylation of proteins of the Smad (Mothers against decapentaplegic homolog) family with the activation of Smad3 as a critical step for uPA regulation in transformed (PDV) keratinocytes or immortalized mouse mammary epithelial cells [9, 10]. The TGF-β pseudoreceptor BMP and Activin Membrane-Bound Inhibitor (Bambi) is capable of inhibiting the pathway [11], whose expression has been recently reported with relevance to chronic obstructive pulmonary disease COPD [12]. In breast cancer MCF-7 cells, TGF-β−induced cellular invasiveness was effectively inhibited by estrogen / estrogen receptor-mediated Smad degradation [13, 14]. In contrast to the majority of studies using cell lines to investigate TGF-β−mediated metastasis, there are very limited data on human breast tumor tissues, therefore, this study focus on the TGF-β pathway and uPA/PAI-1 axis to elucidate the clinical potential directly in human cancer tissues.

Material and Methods

Patient Material

For this study, tissue microarrays (TMAs) of HOPE-fixed tumor specimens from a cohort of 106 patients with breast cancer [15] were subjected to IHC-based detection of TGF−β pathway members and uPA and PAI-1. This cohort were recruited between 2010 and 2011. A summary of the clinicopathological factors are given in Table 1 [15].

Table 1 Distribution of the corresponding relevant clinico-pathological factors of all 107 breast cancer patients [15]

Features	Frequency
All patients	107
Age at surgery	39–98 y (mean 66 y)
pT	
1	34
2	64
3	7
4	1
n.d.	1
pN	
0	55
1	28
2	16
3	4
n.d.	4
Grading	
1	13
2	44
3	50
n.d.	0
HormonR	
ER+PR	53
ER or PR	28
Negative	25
n.d.	1
Her-2/neu	
Positive	15
Negative	91
n.d.	1

ER estrogen receptor, *PR* progesterone receptor, *n.d.* not diagnosed. (as published [15])

Immunohistochemistry (IHC)

Polyclonal rabbit anti-human TGF-β antibody (1:200; Abcam, Cambridge, UK), monoclonal rabbit anti-human Smad3 and (phosphorylated) pSmad3 antibodies (both 1:200; C67H9 and C25A9, respectively, Cell Signaling Technologies, Danvers, USA) and monoclonal mouse anti-human Bambi antibody (1:100; 4e8, eBioscience, San Diego, USA) were applied [16]. Expression of the different TGF-β pathway members was determined qualitatively using a scoring system. Negative expression was defined as the background staining or complete absence of staining signals.

Determination of uPA and PAI-1

The expression of the invasion markers uPA and PAI-1 were measured semi-quantitatively by adapted image analysis in a separate study, where this method has been described in detail [15]. Briefly, Positive staining areas (red color reaction of the chromogen AEC) of the cytoplasmic (uPA and PAI-1) and the

membrane bound (uPA) proteins were extracted by specifying color segments (Adobe Photoshop CS5, Adobe Systems Inc.) from high resolution pictures (magnitude 400×, Leica Digital camera DFC 320) by Leica DM LB2 standard light microscope. After conversion of the colored pictures into black/white negatives, staining intensity of intracellular areas was measured as field integral (Band Leader Application 2.01). The malignant cells were manually chosen at the tumor front based on the original image.

Statistical Evaluation

Correlations between the different effector molecules were evaluated by multiple regression analysis using the best-fit model. Since data of the overall and disease-free survival were not available for this cohort, prognostic factors including age, tumor size (T), lymph node stage (N) grading (G) and steroid hormone receptor status (estrogen and progesterone receptors ER, PR) were incorporated to elucidate mutual interactions and possible clinical relevance (p-values of<0.05 were considered significant). The summary of patients' clinical data is given in Table 1, previously published in [15].

Results

In a total of 88 specimens, antagonistic Bambi and inactive Smad3 molecules showed either no or negligible IHC staining without exceptions (Fig. 1a, b). On the contrary, pSmad3 and TGF-β exhibited varying expression patterns with either homogenous or heterogeneous distribution (Fig. 1d, e). As summarized in Table 1, TGF-β was expressed in 88 % (77/88) of all the tissue samples, 60 % (53/88) were also positive for pSmad3. Additionally, 8 % (7/88) of all the specimens showed pSmad3 protein expression without positive staining for TGF-β. Thus, the vast majority of 96 % (84/88) exhibited an active TGF-β pathway pointing to enhance tumor aggressiveness, with as little as 5 % (4/88) of all the tissues without TGF-β expression. In 88 % (74/84) of these tumors, concomitant high levels of uPA and/or PAI-1 revealed an interdependent, highly significant relationship (Table 2). Additional significant interactions were realized between uPA and G and also between PAI-1 and TGF-β with N and T. Interestingly, TGF-β was also interacting with hormone receptor status, whereby tumor size contributed to PR status (Table 3).

Discussion

To the best of our knowledge, this is the first investigation to examine the complex network of two major systems crucial for invasiveness in human tumor tissues from patients with primary breast cancer. UPA and PAI-1 had been detected by optimized IHC under application of the HOPE-technique, a well-proven tool superior to formalin fixation [17, 18], which prompted additional analyses of specific effector molecules comprising the main parts of the TGF-β signaling pathway. Although the expressions of various proteins belonging to these two pathways were evaluated differently, both methods were based on IHC. Thus, the resulting data were suitable for direct comparisons. The correlation of TGF−β expression with high levels of the two invasion markers, irrespective of phosphorylation of Smad3, corroborates with the existing in vitro data, where TGF-β-regulated enhancement of uPA/PAI-1 occurred through both Smad–dependent and Smad-independent

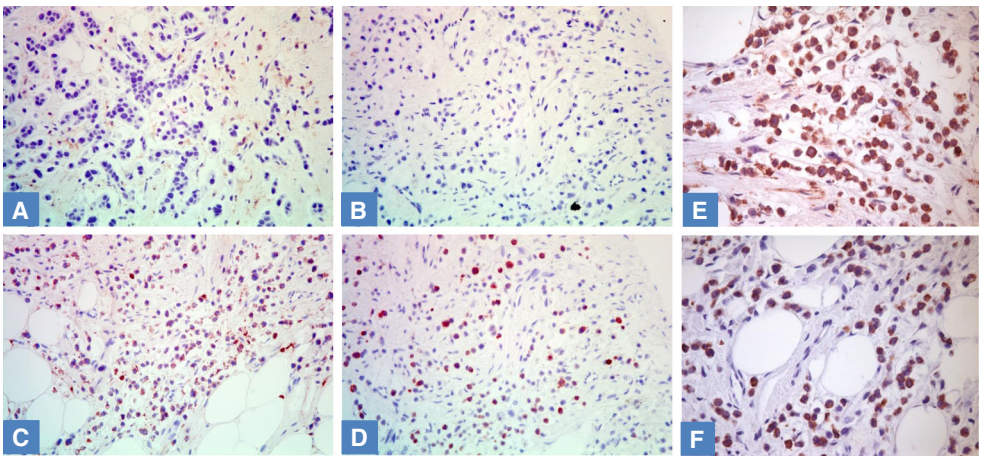

Fig. 1 **a**–**f** A representative sample for immunohistochemical determination of TGF-β signaling and invasion markers. Immunohistochemical expression of TGF−β signaling proteins BAMBI (**a**), Smad3 (**b**), active (phosphorylated) pSmad3 (**c**) and TGF-β (**d**) as well as corresponding invasion biomarkers uPA (**e**) and PAI-1 (**f**) in a representative specimen of primary invasive breast cancer. Both uPA (**e**) and PAI-1 (**f**) were highly expressed (above respective cut-off values [15]). The tissues have been HOPE-fixed and paraffin- embedded. Magnifications are 200fold (**a**–**d**) and 400fold (**e**, **f**)

Table 2 Frequency of key effector proteins (n=88) related to TGF-β pathway or PA system

TGF-ß	Bambi	Smad3	pSmad3	uPA	PAI	Number	Percent
						28	32
						5	6
						13	15
						7	8
				TFG-β +	pSmad3 +	***53***	**60**
						3	3
						0	0
						3	3
						1	1
				TFG-β -	pSmad3 +	***7***	***8***
				Smad-dependent pathway (uPA regulation)		*60*	*68*
						18	20
						2	2
						2	2
						2	2
				TFG-β +	pSmad3 -	***24***	**27**
				Total TGF-β Overexpression		*77*	*88*
				Active TGF-β pathway (tumorpromotion)		*84*	*96*
						1	1
						2	2
						0	0
						1	1
				TFG-β -	pSmad3 -	***4***	***4***
				Inactive TGF-β pathway		*4*	*5*
					Total number	**88**	**100**

Total numbers and percentages are also given for the different combinations and summarized into Smad–dependent and Smad–independent active as well as inactive TGF-β signaling. Green colors = dark green: overexpression of TGF-β-related proteins levels; light green: uPA/PAI-1 above the respective thresholds, indicating high-risk tumors (uPA and/or PAI-1 > cut-off values as provided [15]); red color = absence of IHC staining signal of TGF-β-related proteins; light brown: uPA/PAI-1 levels below respective thresholds, indicating low-risk tumors (both uPA and PAI-1 < cut-off values 143 and 145, respectively [15])

Table 3 Statistically significant relations between TGF β, invasion markers and established clinical factors

Factors	TGF-β	pSmad3	Image uPA	Image PAI-1	ER	PR
Model						
Best fit r2	0.3492	0.1474	0.3742	0.2988	0.5154	0.4756
Adjusted r2	0.2613	0.0322	0.2896	0.2040	0.4499	0.4047
p-value	**0.0002**	0.2577	**<0.0001**	**0.0021**	**<0.0001**	**<0.0001**
Sign. contribution						
TGF-β			**0.0022**		**0.0085**	**0.0174**
pSmad3						
Age		0.0533	0.0670			
T	**0.0002**			0.0694		**0.0075**
N	**0.0225**			**0.0453**		
G			**0.0081**			
PAI-1			**0.0073**			
uPA	**0.0022**			**0.0073**		
ER	**0.0085**					**<0.0001**
PR	**0.0174**				**<0.0001**	

Analysis of correlations between TGF-β and invasion markers uPA or PAI-1 as well as the impact of the corresponding established prognostic tumor characterized by multiple regression model of best fit. *P*-values<0.05 were considered significant. Bold values are statistically significant

pathways in transformed keratinocytes or human MDA-MB-231 cells, respectively [7, 19]. Likewise, pSmad-independent TGF-β activity has been found to promote cell migration in two human breast cancer cell lines [20]. Two major pathways, including mitogen-activated protein kinases (MAPK) ERK and p38 as well as several Phosphotidylinositol-3 kinases (PI3K) are involved in Smad-independent TGF-β signal transduction [21]. More recently, uPA has also been shown to interact with MAPK kinase ERK, whereas PAI-1 was positively correlated with several members of the Pi3K/AKT pathway, pointing to close intercorrelations with TGF-β signaling [22, 23]. In addition, plasmin, a close member of the PA complex, is able to bind and activate TGF-β in conjunction with the specific uPA receptor (uPAR) [24]. Moreover, low-density lipoprotein receptor-related protein 1 (LRP1) was stimulating cellular migration of tumor cells via interaction with PAI-1, identical to TGF-β receptor (V) and tightly involved in the growth regulation induced by TGF-β [25]. Absence of inactive Smad3 protein and antagonistic Bambi points to enhance aggressiveness of most tested breast tumors, which also corroborates Bambi gene suppression in human bladder cancer [26] by suggesting a possible role for Bambi of being tumor-suppressive. However, it remains elusive whether Bambi plays a significant role in normal human breast tissue or not. The mutual impact between TGF-β and steroid hormone receptors corresponds to more recent data reporting crosstalk between estrogen/estrogen receptor and TGF-β / Smad signaling in cell lines [13, 14]. Moreover, the consistent correlations between TGF-β and uPA/PAI-1 and several established prognostic tumor characteristics underline the clinical importance of TGF-β pathway as a promising therapeutic target [27, 28]. Interestingly, uPA and PAI-1, besides there are proven predictive/prognostic value for risk assessment, play additional roles for deregulation of other breast cancer-related signaling pathways [22]. In the former study, both uPA/PAI-1 expression and a panel of signaling molecules were examined based on protein extraction and microarray analysis in breast cancer specimens. In sharp contrast to the present study, there were no consistent correlations between each other as well as with other established clinico-pathological factors except for PAI-1 with nodal status, supporting our hypothesis that the direct determination in HOPE-fixed tumor tissues with focus on the tumor front [29] and combined with image analysis is highly reliable and suitable for enhanced diagnostics in breast cancer. Furthermore, in order to further validate these results, they will be related to corresponding follow-up data for this cohort, as soon as the latter will be available.

In summary, the correlation of TGF-β signaling with uPA/PAI-1 (metastasis) and several other prognostic markers (TNM, ER, PR) strongly suggest a TGF-β-dependency of breast cancer. Thus, these results may contribute to the improvement of anticancer therapy (such as Anti-TGF-β) with targeted approaches.

Acknowledgments The authors would like to thank Jasmin Tiebach, Maria Lammers and Stefanie Fox for their excellent technical support.

Conflict of Interest There is no conflict of interest for any of the authors.

Ethics The research protocol has been approved by the Ethics committees of Bad Segeberg (39/09) and of the University of Lübeck (07–157).

Authors' Contributions DSL carried out the semi-quantitative image analyses, statistical evaluation and drafted the manuscript. SM carried out and evaluated the staining's of the TGF-β pathway members and was involved in the drafting of the manuscript. UH and OB were responsible for the surgical part and clinical data. WS and RS provided the surgical tissue material following routine pathology. MR has contributed in writing and finalizing the manuscript. EV was responsible for the histopathological aspects. TG conceived of the study and was involved in drafting the manuscript. All authors have read and approved the final manuscript.

References

1. Welch DR, Steeg PS, Rinker-Schaeffer CW (2000) Molecular biology of breast cancer metastasis. Genetic regulation of human breast carcinoma metastasis. Breast Cancer Res 2:408–412
2. Lyer S, Wang Z-G, Akhtari M, Zhao W, Seth P (2005) Targeting TGFβ signaling for cancer therapy. Cancer Biol Ther 4(3):261–266
3. Malinowsky K, Böllner C, Hipp S, Berg D, Schmitt M, Becker KF (2010) UPA and PAI-1 analysis from fixed tissues—new perspectives for a known set of predictive markers. Curr Med Chem 17: 4370–4377 (Review)
4. Jo M, Eastman BM, Webb DL, Stoletov K, Klemke R, Gonias SL (2010) Cell signaling by urokinase-type plasminogen activator receptor induces stem cell-like properties in breast cancer cells. Cancer Res 70(21):8948–8958
5. Harbeck N, Schmitt M, Vetter M, Krol J, Paepke D, Uhlig M, Paepke S, Jänicke F, Geurts-Moespot A, von Minckwitz G, Sweep F, Thomssen C (2008) Prospective biomarker trials Chemo NO and NNBC-3 Europe validate the clinical utility of invasion markers uPA and PAI-1 in node-negative breast cancer. Breast Care 3(suppl 2):11–15
6. Kreienberg R, Albert U-S, Follmann M, Kopp I, Kühn T, Wöckel A, Zemmler T (2012) Interdisziplinäre S-3 Leitlinie für Diagnostik, Therapie und Nachsorge des Mammakarzinoms. Published by German Cancer Society e.V
7. Shiou S-R, Datta PK, Dhawan P, Law BK, Yingling JM, Dixon DA, Beauchamp RD (2006) Smad4-dependent regulation of urokinase plasminogen activator secretion and RNA stability associated with invasiveness by autocrine and paracrine transforming growth factor-β. J Biol Chem 281:33971–33981
8. Song X, Thalacker FW, Nilsen-Hamilton M (2012) Synergistic and multidimensional regulation of plasminogen activator inhibitor type 1 expression by transforming growth factor type β and epidermal growth factor. J Biol Chem 287:12520–12528
9. Kohn EA, Yang YA, Du Z, Nagano Y, Van Schyndle CM, Herrmann MA, Heldman M, Chen J-Q, Stuelten CH, Flanders KC (2012) Wakefield LM (2012) Biological responses of TGF-β in the mammary epithelium show a complex dependency on Smad3 gene dosage with important implications for tumor progression. Mol Cancer Res 10(10):1389–1399
10. Kocic J, Bugarski D, Santibanez JF (2012) Smad3 is essential for transforming growth factor-β1-induced urokinase type plasminogen activator expression and migration in transformed keratinocytes. Eur J Cancer 48:1550–1557

11. Onichtchouk D, Chen YG, Dosch R, Gawantka V, Delius H, Massaque J, Niehrs C (1999) Silencing of TGF-beta signaling by the pseudoreceptor BAMBI. Nature 401:480–485
12. Drömann D, Rupp J, Rohmann K, Osbahr S, Ulmer A, Marwitz S, Röschmann K, Abdullah M, Schultz H, Vollmer E, Zabel P, Dalhoff K, Goldmann T (2010) The TGF-beta- pseudoreceptor BAMBI is strongly expressed in COPD lungs and regulated by nontypeable Haemophilus influenzae. Respir Res 11:67
13. Ito I, Hanyu A, Wayama M, Goto N, Katsuno N, Kawasaki S, Nakajima Y, Kajiro M, Komatsu Y, Fujimura A, Hirota R, Murayama A, Kimura K, Imamura T, Yanagisawa J (2010) Estrogen inhibits transforming growth factor β signaling by promoting Smad2/3 degradation. J Biol Chem 285:14747–14755
14. Goto N, Hiyoshi H, Ito I, Tsuchiya M, Nakajima Y, Yanagisawa J (2011) Estrogen and antiestrogens alter breast cancer invasiveness by modulating the transforming growth factor-β signaling pathway. Cancer Sci 102:1501–1508
15. Lang DS, Heilenkötter U, Schumm W, Behrens O, Simon R, Vollmer E, Goldmann T (2013) Optimized immunohistochemistry in combination with image analysis: a reliable alternative to quantitative ELISA determination of uPA and PAI-1 for routine risk group discrimination in breast cancer. Breast. doi:10.1016/j.breast.2012.12.011
16. Schulz H, Kähler D, Branscheid D, Vollmer E, Zabel P, Goldmann T (2008) TKTL1 is overexpressed in a large portion of non-small cell lung cancer specimens. Diagn Pathol 3:35
17. Vollmer E, Galle J, Lang DS, Loeschke S, Schultz H, Goldmann T (2006) The HOPE technique opens up a multitude of new possibilities in pathology. Rom J Morphol Embryol 47(1):15–19 (Review)
18. Marwitz S, Abdullah M, Vock C, Fine JS, Visvanathan S, Gaede KI, Hauber HP, Zabel P, Goldmann T (2011) HOPE-BAL: improved molecular diagnostics by application of a novel technique for fixation and paraffin embedding. J Histochem Cytochem 59(6):601–614
19. Tobar N, Villar V, Santibanez JF (2010) ROS-NFkB mediates TGF-β1-induced expression of urokinase-type plasminogen activator, matrix metalloproteinase-9 and cell invasion. Mol Cell Biochem 340: 195–202
20. Giehl K, Imamichi Y, Menke A (2007) Smad4-independent TGF–β signaling in tumor cell migration. Cells Tissues Organs 185:123–130
21. Wakefield LM, Roberts AB (2002) TGF-b signaling: positive and negative effects on tumorigenesis. Curr Opin Genet Dev 12:22–29 (Review)
22. Wolff C, Malinoswky K, Berg D, Schragner K, Schuster T, Walch A, Bronger H, Höfler H, Becker K-F (2011) Signalling networks associated with urokinase-type plasminogen activator (uPA) and ist inhibitor PAI-1 in breast cancer tissues: new insights from protein microarray analysis. J Pathol 223:54–63
23. Malinowsky K, Wolff C, Berg D, Schuster T, Walch A, Bronger H, Mannsperger H, Schmidt C, Korf U, Höfler H, Becker K-F (2012) uPA and PAI-1-related signaling pathways differ between primary breast cancers and lymph node metastases. Trans Oncol 5:98–104
24. Czekay R-P, Wilkins-Port CE, Higgins SP, Freytag J, Overstreet JM, Klein RM, Higgins CE, Samarakoon R, Higgins PJ (2011) PAI-1: an integrator of cell signaling and migration. Int J Cell Biol 2011:1–9 (Review)
25. Huang SS, Ling T-Y, Tseng W-F, Huang Y-W, Tang F-M, Leal SM, Huang JS (2003) FASEB J 17:2068–2080
26. Khin SS, Kitazawa R, Win N, Aye TT, Mori K, Kondo T, Kitazawa S (2009) BAMBI gene is epigenetically silenced in subset of high-grade bladder. Int J Cancer 125:328–338
27. Ganapathy V, Rongrong G, Grazioli A, Xie W, Banach-Petrosky W, Kang Y, Lonning S, McPherson J, Yingling JM, Biswas S, Mundy GR, Reiss M (2010) Targeting the transforming growth factor–β pathway inhibits human basal-like breast cancer metastasis. Mol Cancer 9:122
28. Liu J, Liao S, Diop-Frimpong B, Chen W, Goel S, Naxerova K, Ancukiewicz M, Boucher Y, Jain RK, Xu L (2012) TGF-β blockage improves the distribution and efficiacy of therapeutics in breast carcinoma by normalizing the tumor stroma. PNAS 109:16618–16623
29. Matise LA, Palmer TD, Ashby WJ, Nashabi A, Chytil A, Aakre M, Pickup MW, Gorska AE, Zijlstra A, Moses HL (2012) Lack of transforming growth factor-β signaling promotes collective cancer cell invasion through tumor-stromal crosstalk. Breast Cancer Res 14: R98