ELSEVIER

European Journal of Cancer 40 (2004) 2417–2423

European Journal of Cancer

www.ejconline.com

Thrombospondin-1 expression in relation to p53 status and VEGF expression in human breast cancers

Barbro Linderholm *, Eva Karlsson, Sigrid Klaar, Thomas Lindahl, Anna-Lena Borg, Göran Elmberger, Jonas Bergh

Department of Oncology and Pathology, CCK Cancer Centrum Karolinska, Karolinska Institutet and Hospital, SE-171 76 Stockholm, Sweden

Received 27 February 2004; received in revised form 28 June 2004; accepted 29 July 2004
Available online 11 September 2004

Abstract

The aim of the present study was to study the expression and relationship of potential angiogenic factors. Paraffin-embedded tumour sections from 261 breast cancer patients were stained immunohistochemically for thrombospondin (TSP-1) expression. p53 status was previously determined by cDNA-based sequencing, and vascular endothelial growth factor (VEGF) protein expression had been previously analysed using an immunoassay. 241 cancers (92%) had detectable levels of TSP-1. No associations between TSP-1 and p53 status or VEGF were found. No correlations between TSP-1 and relapse-free ($P = 0.3$), breast cancer-corrected ($P = 0.2$) or overall survival ($P = 0.5$) were found. A correlation was found for patients with p53 mutations, but negative p53 expression, with higher VEGF levels ($P = 0.009$), but there was no correlation between this p53 group and those with low TSP-1 levels ($P = 0.2$). In conclusion, TSP-1 expression was not prognostic and was not associated with neither p53-status or VEGF expression.

Keywords: Breast cancer; Thrombospondin; p53; Angiogenesis; Vascular endothelial growth factor

1. Introduction

Thrombospondins are a family of five glycoproteins. Thrombospondin-1 (TSP-1) is a high-molecular-weight, multifunctional glycoprotein that is part of the extracellular matrix, and was first described as a product of platelets [1,2]. TSP-1 has been reported to be active in diverse biological processes, such as embryonic development, tissue differentiation, nerve outgrowth and regeneration, inflammation, coagulation, fibrinolysis, angiogenesis, as well as tumour growth and metastasis [3]. In various neoplastic cell lines, the levels of TSP-1 expression show an inverse correlation with malignant progression [4–7].

An inverse correlation between TSP-1 and immunohistochemical p53 overexpression has been reported in a variety of malignant tissues, including bladder, colorectal, ovarian and prostate cancers [8–11]. However, thrombospondin has been linked with both a stimulatory [12–14] and inhibitory [4,5] role in tumour invasiveness and progression. TSP has been reported to be present in normal breast tissues, while higher levels of TSP have been observed in breast cancer tissues [15–17]. Other studies have shown TSP to be absent in normal breast tissues, but present in breast cancer tissues [13].

Wild-type p53 can prevent the proliferation of DNA-damaged cells by either causing a cell cycle arrest or activating apoptosis [18]. Vascular endothelial growth factor (VEGF) is a mitogen for endothelial cells, and may be a crucial mediator for neovascularisation in human tumours [19,20]. An association between mutant p53

* Corresponding author. Tel.: +46 8 517 762 79; fax: +46 8 517 795 24.
E-mail address: barbro.linderholm@cck.ki.se (B. Linderholm).

0959-8049/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ejca.2004.07.026

and VEGF expression and a poor prognosis for breast cancer patients has been reported [21].

The aim of this study was to determine any associations between TSP-1 expression and p53 status and/or VEGF expression in primary invasive breast cancer patients. Secondary aims were to investigate the clinical relevance of TSP-1 expression, alone and in combination, with p53 status and/or VEGF expression.

2. Patients and methods

2.1. Study materials and patient population

The patients in our present study were derived from a population-based cohort of 315 primary breast cancer patients who underwent breast cancer surgery during 1987–1989 in Uppsala County, Sweden, and from whom frozen tumour material was available. These patients have been described in detail in [22].

All breast cancer samples were sent directly, unfixed and fresh, from the operation theatre to the Department of Pathology for routine histopathological examination, analysis of oestrogen and progesterone receptor status, ploidy and S-phase fraction. The median patient age at diagnosis was 63 years (range 28–94 years). The median tumour size was 20 mm (range 3–130 mm). Lymph node metastases were detected in 97 (31%) patients. 261 of the 315 tumours, were assessable for TSP-1 (Table 1) and, as previously described, 224 tumours were assessable for VEGF, 311 tumours were evaluable for sequence-based analysis of p53 and 312 tumours were evaluable for immunohistochemical (IHC) analysis of p53 protein [23–25].

Table 1
Comparison between the cohort of 315 patients from January 1, 1987 to December 31, 1989 and the selected 261 patients

Clinical parameters	261 patient group	315 patient group
Tumour size (mm)		
Median (range)	20 (3–130)	20 (2–130)
Mean	22	22
Lymph node[a]		
Negative	171	206
Positive	81	97
Positive/total	0.32	0.32
S-phase fraction		
Low	193	235
High	54	62
Unknown	14	18
Oestrogen receptor (positive > 0.1 fmol/μg DNA)		
Negative	54	64
Positive	202	244
Unknown	5	7
Progesterone receptor (positive > 0.1 fmol/μg DNA)		
Negative	38	49
Positive	218	259
Unknown	5	7

[a] Some data are missing.

2.2. Treatment

All patients underwent surgery either by sector resection or modified radical mastectomy. Both procedures were combined with axillary lymph node dissection, except in 13 patients in whom age (older) or serious concomitant disease contraindicated an axillary dissection. Loco-regional radiotherapy was normally given to patients with node-positive disease or tumours larger than 20 mm and located in the medial or central area of the breast, except in the presence of metastatic disease, serious concomitant disease or older age. Postoperative radiotherapy was given as part of a randomised study to those operated upon with a sector resection from October 1981 to September 1988. After closure of the study, it was recommended that all patients routinely undergo a sector resection [26].

Systemic adjuvant therapy was offered to all node-positive patients. In general, premenopausal patients received adjuvant chemotherapy, and postmenopausal node-positive patients received adjuvant tamoxifen endocrine therapy for 2–5 years.

2.3. Follow-up

All patients treated for breast cancer in Uppsala County were routinely seen on a regular outpatient basis for 5–10 years. Routine follow-up consisted of clinical examination. Blood tests and X-ray procedures were performed when clinically indicated. Patients 40–74 years of age were also offered participation in a population-based mammography-screening programme.

2.4. Immunohistochemical staining for TSP-1

Stored paraffin sections from 1999 were used. In 1999, a small piece of the fresh frozen tumour tissue was removed, thawed, fixed and sectioned. Storage of paraffin sections may *lead* to a varying degree of decreased immunoreactivity for several antibodies [27]. This may be compensated for by optimising the antigen retrieval (AR) protocol [27]. The paraffin sections we used were first tested for changes in immunoreactivity using citrate buffer or boric acid for antigen retrieval. This resulted in equal specific staining, but the intensity of staining was higher when using boric acid than that obtained using citrate buffer (G. Elmberger, not shown). Four μm paraffin-embedded tumour sections were deparaffinised in xylene and rehydrated in graded alcohol. Antigen retrieval was achieved by heating the slides in 0.2 M boric acid, pH 7.2 in 85 °C for 16 h [27]. The immunostaining was

done in a DAKO TechMate Instrument, (DAKO, Denmark), an automated Immunohistochemistry instrument. We used the manufacturer's diaminobenzidine (DAB) detection kit, (DAKO ChemMate Detection Kit, Peroxidase/DAB, Rabbit/Mouse for the DAKO TechMate instruments), that includes biotin-labelled secondary antibodies directed against mouse/rabbit immunoglobulins, peroxidase-conjugated streptavidin molecules and DAB as the localisation reagents. This allows each biotinylated antibody molecule to react with several peroxidase-conjugated streptavidin molecules. For primary antibody, we used a mouse-monoclonal antibody, TSP-1, clone p12 (Immunotech, Marseille, France) [28–30]. The primary antibody diluted to 1/20 gave the optimal staining reaction.

Paraffin-embedded human tonsil sections were used as positive controls [28,31]. Negative controls were performed by omitting the primary antibody, TSP-1, from the paraffin-embedded tonsils. The intensity of staining in the tumour cells, was scanned and arbitrarily graded; (0, no staining noted; 1, low intensity of staining in >10% of the tumour cells; 2, medium intensity of staining in >10% of the tumour cells; 3, high intensity of staining in >10% of the tumour cells). All slides were viewed and scored by one pathologist without knowledge of patients' clinical outcome.

2.5. p53 status by cDNA-based sequencing

Sequence-based analysis of the complete coding region of p53 has been reported previously in [22,25]. The entire *TP53* gene was analysed.

2.6. p53 status by immunohistochemistry

p53 status in tumours was analysed by IHC on paraffin sections using the monoclonal mouse antibody, Pab1801, as earlier described in [25].

2.7. Vascular endothelial growth factor

VEGF was analysed using an enzyme-linked immunosorbent assay (ELISA), as previously described in [23,24]. VEGF content was expressed as pg protein/mg of total cytosolic protein.

2.8. Statistical methods

Overall survival (OS), breast cancer-corrected survival (BCCS), and relapse-free survival (RFS) were analysed by the Kaplan–Meier method. The log-rank-test was employed for the analysis of differences between groups. All *P*-values were derived from two-tailed tests. For OS all deaths were counted as events. For BCCS, only deaths from breast cancer were considered as events. Events for estimation of RFS were all breast cancer relapses, (not including new contralateral breast cancer) or death from breast cancer. Women without recurrence were censored at the time of their latest follow-up. Relative hazards of dying of breast cancer were estimated by use of Cox's proportional hazards method in a univariate model. Analyses were done with age and tumour size as continuous variables, and other factors as dichotomous variables. p53 status was used as positive *versus* negative and the median value of VEGF was used as the cut-off value, except in the analysis of variance (ANOVA) analysis, where VEGF was used as a continuous variable. Distribution of TSP-1 in the different subgroups was compared by the χ^2 test. Multivariate analysis was not performed, as TSP-1 was not a statistically significant factor in the univariate analysis. The analyses were carried out using the JMP software for PC from the SAS Institute (Cortex, AB, Solna, Sweden) and with the PHREG procedure in SAS for IBM.

3. Results

3.1. Clinical outcome

A total of 106 recurrences have been registered, and 137 patients have died during the follow-up period, a median time of 122 months and maximum of 153 months. For 74 patients, the cause of death was breast cancer.

3.2. Distribution of TSP-1

Stored paraffin sections could be obtained from 273 of the 315 tumours and 12 of these 273 sections were not assessable for TSP-1 due to insufficient material or staining, leaving 261 tumours. Staining for TSP-1 was observed primarily in the tumour, endothelial and plasma cells. Staining was located in the luminal and apical portions of the malignant ductal epithelium. In poorly differentiated tumour cells, the cytoplasmic staining was more diffuse for TSP-1 expression. No evidence of TSP-1 was seen in the stroma, or basement membrane of normal ductules. Staining was also seen in normal breast cells, when they were present in the slides. In normal breast cells, the staining was clear, distinct and found in the apical portion of the cells (Fig. 1(a)–(c)). Overall, 241 (92%) had detectable levels of TSP-1 (arbitrarily graded 0-3), while 20 tumours were negative. TSP-1 expression was not related to the patient's nodal-status.

3.3. Correlation between TSP-1 and p53 or VEGF

No significant correlations were found between TSP-1 and p53 status or VEGF. In patients with p53

mutations, determined by cDNA-based sequencing, but with negative p53 status by IHC ($n = 10$), we found a correlation with high VEGF expression ($P = 0.009$). This was not seen between this p53 group and those with low TSP-1 expression ($n = 21$) ($P = 0.2$) (Tables 2 and 3). In the group with TSP-1-negative tumours (TSP-1 = 0) and p53 mutations, two out of seven (29%) mutations consisted of point mutations compared with 29 out of 49 (59%) for the TSP-1-positive tumours (TSP-1 = 1–3) with p53 mutations.

3.4. TSP-1 and patient outcome

No correlation was found between TSP-1 expression (graded 0–3) and survival, OS ($P = 0.5$), BCCS ($P = 0.2$), or RFS ($P = 0.3$). Evaluation of TSP and VEGF as continuous variables were not correlated with survival (data not shown).

When the total patient group was stratified with regard to their p53 status, no correlation was found between TSP-1 and OS in the groups with wild-type ($P = 0.4$) or mutated p53 ($P = 0.8$). In subgroup analyses, node-negative patients without systemic adjuvant treatment, or node-positive patients receiving adjuvant chemotherapy or endocrine therapy, no differences in survival were seen according to their TSP-1 status.

4. Discussion

The angiogenic pathway and its regulation has resulted in many publications, including studies on TSP and p53. However, suboptimal, methods, such as IHC, have been used for the determination of p53 status [25,32–39].

To our knowledge, this study, for the first time, compares *TSP-1 expression* with p53 status according to the complete gene sequence using population-derived breast cancer samples.

In contrast to other studies, we found no evidence of TSP-1 staining in the stroma or at the basement membrane of the ductules [10,13,17,40,41]. Different fixation

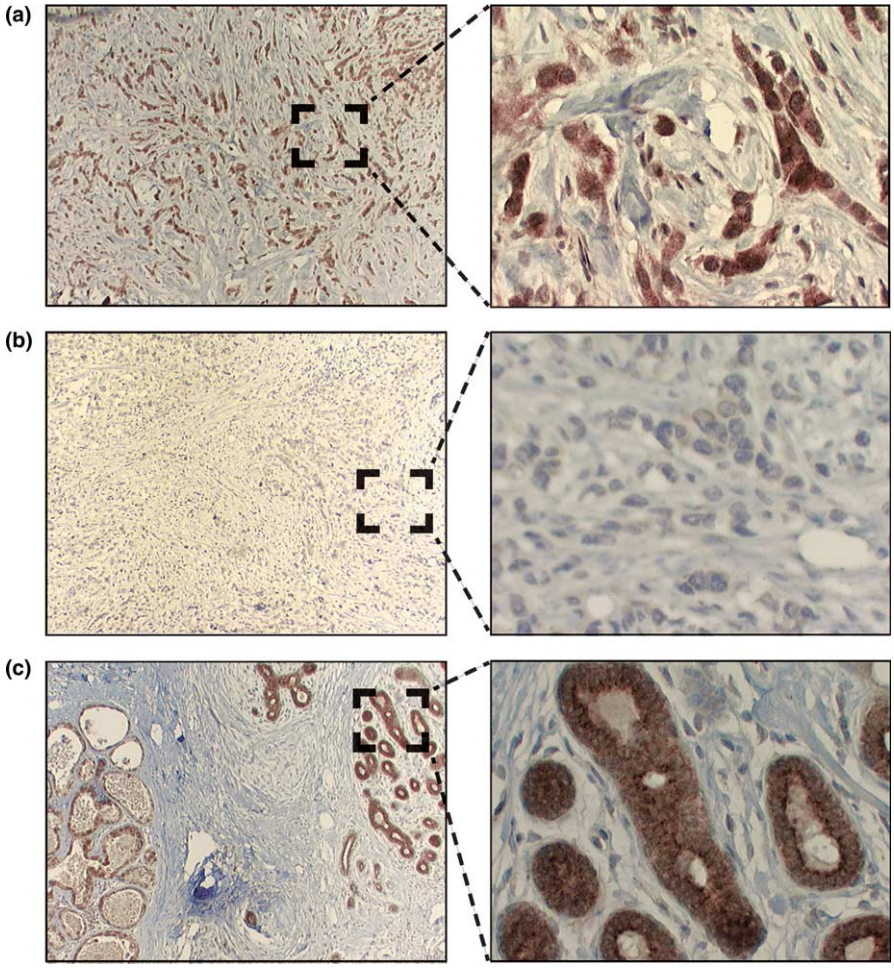

Fig. 1. (a) Immunohistochemical stains for thrombospondin-1 (TSP-1). (a) Breast cancer tissue showing 3+ staining. (b) Breast cancer tissue showing negative staining (level 0). (c) Normal hyperplastic breast tissue showing strong immunoreactivity.

Table 2
Distribution of low- and high-TSP-1 levels in subgroups defined by immunohistochemical and sequence-based p53 status

	p53-wt/IHC negative $n = 187$	p53-wt/IHC positive $n = 15$	p53-mut/IHC negative $n = 21$	p53-mut/IHC positive $n = 34$		
TSP-1-low						
%	61	53	76	59		
(*n*)	(114)	(8)	(16)	(20)	158	
						$P = 0.5$
TSP-1-high						
%	39	47	24	41		
(*n*)	(73)	(7)	(5)	(14)	99	
			$P = 0.2$*		257	

TSP-1-low, groups 0 and 1; TSP-1-high, groups 2 and 3.
* No correlation was found between high TSP-1 and p53 mutations by sequencing but negative IHC.

Table 3
Distribution of low- and high-VEGF levels in subgroups defined by immunohistochemical and sequence-based p53 status

	p53-wt/IHC negative	p53-wt/IHC positive	p53-mut/IHC negative	p53-mut/IHC positive		
VEGF-low						
%	57	31	20	31		
(*n*)	(98)	(4)	(2)	(8)	112	
						$P = 0.005$
VEGF-high						
%	43	69	80	69		
(*n*)	(75)	(9)	(8)	(18)	110	
VEGF-mean						
pg/mg total protein	488	427	1212	928		
Range	8–6199	37–1121	103–2604	71–6725		$P = 0.009$
(*n*)	(173)	(13)	(10)	(26)	222	

VEGF, vascular endothelial growth factor.
VEGF-low, below median value; VEGF-high, above median value.

procedures, TSP antibodies or IHC techniques are possible explanations for the recorded differences. From our IHC results, it appears that TSP-1 expression is more well-organised in normal cells than in tumour cells.

In our study, no associations between *TSP-1 and VEGF expression and p53-status* were found. These findings are consistent with recently reported data in breast cancer patients [42], but in contrast to data from studies of bladder, colorectal, epithelial ovarian and prostate cancers, where an inverse correlation between TSP-1 expression and increased IHC p53 expression has been reported [8–11]. One can speculate that the use of different methods in these studies for the determination of TSP and p53 status may partly *explain* the conflicting results.

No correlation was found between TSP-1 expression and survival in the total patient group, nor when patients were stratified with regard to their p53 status. A possible explanation for these results is that TSP-1 is only one of many factors that are known to be involved in angiogenesis.

We and others have previously described suboptimal results for the determination of p53 using protein-based methods like IHC compared with sequencing [25,32–39]. False-negative p53 IHC results may arise as a consequence of the mutation type, and false-positive results due to activation of wild-type p53 [25]. As earlier published we found statistically significant higher VEGF expression ($P = 0.009$) [21], but no correlation in those with low TSP-1 values ($P = 0.2$) in the group with sequence-positive tumours (proven to have p53 mutation), which were p53-negative by IHC.

Dameron and colleagues [43] showed in a preclinical setting that normal p53 activates the *TSP-1* gene at the transcriptional level. However, our data does not support this, therefore we propose that other mechanisms of TSP-1 regulation, in addition to p53, must be operating in these cells. Angiogenesis is regulated by *several* factors in a tumour and the expression of these may differ depending on the tumour's biological characteristics, as well as changes within the tumour and surrounding tissue(s) over time. One can speculate further that angiogenic stimuli in the tumour, such as *hypoxia*, may predominate over existing inhibitors of angiogenesis, such as TSP-1, and this might explain the lack of a clinical correlation for TSP-1.

Data from the literature indicates that the inhibition of angiogenesis by TSP-1 is mediated by two independent

regions of the molecule [44]. The amino-terminal end contains a type 1 repeat which inhibits angiogenesis stimulated by the basic fibroblast growth factor (bFGF), whereas a second region near the carboxy-terminus exerts its anti-angiogenetic effect by inhibiting either bFGF or VEGF-driven angiogenesis. Two other studies indicate that TSP-1 may exert its anti-angiogenetic effect by inducing endothelial cells to undergo apoptosis [45,46]. There are reports indicating that TSP-1 might affect angiogenesis in opposing directions, depending on which domain of the molecule is active [47], or alternatively, depending on the nature and number of TSP-1 receptors that are present on the endothelial cells [3,48]. TSP-1 therefore possibly has the capacity to both stimulate and inhibit endothelial cell proliferation and angiogenesis. This, and as described above, the use of suboptimal methods for the determination of p53, may explain the conflicting reports on the effects of TSP-1 and its correlation to p53.

In our study, TSP-1 was not associated with p53-status, VEGF expression or patients' survival.

Conflict of interest statement

None declared.

Acknowledgements

The study was supported by grants from the Swedish Cancer Society, Stockholm Cancer Society and King Gustav the fifth jubilee foundation.

We are grateful to Dr. Anders Lindgren for collecting the tumour bank material and to Helena Hermelin for tumour handling and sectioning. Many thanks to Anna-Lena Borg for running the IHC staining procedure.

References

1. Lawler J. The structural and functional properties of thrombospondin. *Blood* 1986, **67**(5), 1197–1209.
2. Baenziger NL, Brodie GN, Majerus PW. Isolation and properties of a thrombin-sensitive protein of human platelets. *J Biol Chem* 1972, **247**(9), 2723–2731.
3. Bornstein P. Diversity of function is inherent in matricellular proteins: an appraisal of thrombospondin 1. *J Cell Biol* 1995, **130**(3), 503–506.
4. Weinstat-Saslow DL, Zabrenetzky VS, VanHoutte K, Frazier DD, Roberts DD, Steeg PS. Transfection of thrombospondin 1 complementary DNA into a human breast carcinoma cell line reduces primary tumor growth, metastatic potential, and angiogenesis. *Cancer Res* 1994, **54**(24), 6504–6511.
5. Zabrenetzky V, Harris CC, Steeg PS, Roberts DD. Expression of the extracellular matrix molecule thrombospondin inversely correlates with malignant progression in melanoma, lung and breast carcinoma cell lines. *Int J Cancer* 1994, **59**(2), 191–195.
6. Hsu SC, Volpert OV, Steck PA, Mikkelsen T, Polverini PJ, Rao S, *et al*. Inhibition of angiogenesis in human glioblastomas by chromosome 10 induction of thrombospondin-1. *Cancer Res* 1996, **56**(24), 5684–5691.
7. Grant SW, Kyshtoobayeva AS, Kurosaki T, Jakowatz J, Fruehauf JP. Mutant p53 correlates with reduced expression of thrombospondin-1, increased angiogenesis, and metastatic progression in melanoma. *Cancer Detect Prev* 1998, **22**(3), 185–194.
8. Alvarez AA, Axelrod JR, Whitaker RS, Isner PD, Bentley RC, Dodge RK, *et al*. Thrombospondin-1 expression in epithelial ovarian carcinoma: association with p53 status, tumor angiogenesis, and survival in platinum-treated patients. *Gynecol Oncol* 2001, **82**(2), 273–278.
9. Tokunaga T, Nakamura M, Oshika Y, Tsuchida T, Kazuno M, Fukushima Y, *et al*. Alterations in tumour suppressor gene p53 correlate with inhibition of thrombospondin-1 gene expression in colon cancer cells. *Virchows Arch* 1998, **433**(5), 415–418.
10. Grossfeld GD, Ginsberg DA, Stein JP, Bochner BH, Esrig D, Groshen S, *et al*. Thrombospondin-1 expression in bladder cancer: association with p53 alterations, tumor angiogenesis, and tumor progression. *J Natl Cancer Inst* 1997, **89**(3), 219–227.
11. Mehta R, Kyshtoobayeva A, Kurosaki T, Small EJ, Kim H, Stroup R, *et al*. Independent association of angiogenesis index with outcome in prostate cancer. *Clin Cancer Res* 2001, **7**(1), 81–88.
12. Wang TN, Qian X, Granick MS, Solomon MP, Rothman VL, Berger DH, *et al*. Thrombospondin-1 (TSP-1) promotes the invasive properties of human breast cancer. *J Surg Res* 1996, **63**(1), 39–43.
13. Wong SY, Purdie AT, Han P. Thrombospondin and other possible related matrix proteins in malignant and benign breast disease. An immunohistochemical study. *Am J Pathol* 1992, **140**(6), 1473–1482.
14. Tuszynski GP, Gasic TB, Rothman VL, Knudsen KA, Gasic GJ. Thrombospondin, a potentiator of tumor cell metastasis. *Cancer Res* 1987, **47**(15), 4130–4133.
15. Pratt DA, Miller WR, Dawes J. Thrombospondin in malignant and non-malignant breast tissue. *Eur J Cancer Clin Oncol* 1989, **25**(2), 343–350.
16. Dawes J, Clezardin P, Pratt DA. Thrombospondin in milk, other breast secretions, and breast tissue. *Semin Thromb Hemost* 1987, **13**(3), 378–384.
17. Clezardin P, Frappart L, Clerget M, Pechoux C, Delmas PD. Expression of thrombospondin (TSP1) and its receptors (CD36 and CD51) in normal, hyperplastic, and neoplastic human breast. *Cancer Res* 1993, **53**(6), 1421–1430.
18. Sjostrom J, Bergh J. How apoptosis is regulated, and what goes wrong in cancer. *BMJ* 2001, **322**(7301), 1538–1539.
19. Ferrara N. Vascular endothelial growth factor. *Eur J Cancer* 1996, **32A**(14), 2413–2422.
20. Kim KJ, Li B, Winer J, Armanini M, Gillett N, Phillips HS, *et al*. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth *in vivo*. *Nature* 1993, **362**(6423), 841–844.
21. Linderholm BK, Lindahl T, Holmberg L, Klaar S, Lennerstrand R, Henriksson R, *et al*. The expression of vascular endothelial growth factor correlates with mutant p53 and poor prognosis in human breast cancer. *Cancer Res* 2001, **61**(5), 2256–2260.
22. Bergh J, Norberg T, Sjogren S, Lindgren A, Holmberg L. Complete sequencing of the p53 gene provides prognostic information in breast cancer patients, particularly in relation to adjuvant systemic therapy and radiotherapy. *Nat Med* 1995, **1**(10), 1029–1034.
23. Linderholm B, Grankvist K, Wilking N, Johansson M, Tavelin B, Henriksson R. Correlation of vascular endothelial growth factor content with recurrences, survival, and first relapse site in primary node-positive breast carcinoma after adjuvant treatment. *J Clin Oncol* 2000, **18**(7), 1423–1431.

24. Linderholm B, Tavelin B, Grankvist K, Henriksson R. Vascular endothelial growth factor is of high prognostic value in node-negative breast carcinoma. *J Clin Oncol* 1998, **16**(9), 3121–3128.
25. Sjogren S, Inganas M, Norberg T, Lindgren A, Nordgren H, Holmberg L, *et al*. The p53 gene in breast cancer: prognostic value of complementary DNA sequencing *versus* immunohistochemistry. *J Natl Cancer Inst* 1996, **88**(3–4), 173–182.
26. Liljegren G, Holmberg L, Bergh J, Lindgren A, Tabar L, Nordgren H, *et al*. 10-Year results after sector resection with or without postoperative radiotherapy for stage I breast cancer: a randomized trial. *J Clin Oncol* 1999, **17**(8), 2326–2333.
27. Wester K, Wahlund E, Sundstrom C, Ranefall P, Bengtsson E, Russell PJ, *et al*. Paraffin section storage and immunohistochemistry. Effects of time, temperature, fixation, and retrieval protocol with emphasis on p53 protein and MIB1 antigen. *Appl Immunohistochem Mol Morphol* 2000, **8**(1), 61–70.
28. Clezardin P, McGregor JL, Lyon M, Clemetson KJ, Huppert J. Characterization of two murine monoclonal antibodies (P10, P12) directed against different determinants on human blood platelet thrombospondin. *Eur J Biochem* 1986, **154**(1), 95–102.
29. Legrand C, Dubernard V, Kieffer N, Nurden AT. Use of a monoclonal antibody to measure the surface expression of thrombospondin following platelet activation. *Eur J Biochem* 1988, **171**(1–2), 393–399.
30. McLaren KM. Immunohistochemical localisation of thrombospondin in human megakaryocytes and platelets. *J Clin Pathol* 1983, **36**(2), 197–199.
31. Girard JP, Baekkevold ES, Yamanaka T, Haraldsen G, Brandtzaeg P, Amalric F. Heterogeneity of endothelial cells: the specialized phenotype of human high endothelial venules characterized by suppression subtractive hybridization. *Am J Pathol* 1999, **155**(6), 2043–2055.
32. Tolbert DM, Noffsinger AE, Miller MA, DeVoe GW, Stemmermann GN, Macdonald JS, *et al*. p53 immunoreactivity and single-strand conformational polymorphism analysis often fail to predict p53 mutational status. *Mod Pathol* 1999, **12**(1), 54–60.
33. Kressner U, Inganas M, Byding S, Blikstad I, Pahlman L, Glimelius B, *et al*. Prognostic value of p53 genetic changes in colorectal cancer. *J Clin Oncol* 1999, **17**(2), 593–599.
34. Smith DR, Ji CY, Goh HS. Prognostic significance of p53 overexpression and mutation in colorectal adenocarcinomas. *Br J Cancer* 1996, **74**(2), 216–223.
35. Dix B, Robbins P, Carrello S, House A, Iacopetta B. Comparison of p53 gene mutation and protein overexpression in colorectal carcinomas. *Br J Cancer* 1994, **70**(4), 585–590.
36. Mineta H, Borg A, Dictor M, Wahlberg P, Akervall J, Wennerberg J. p53 mutation, but not p53 overexpression, correlates with survival in head and neck squamous cell carcinoma. *Br J Cancer* 1998, **78**(8), 1084–1090.
37. Duddy PM, Hanby AM, Barnes DM, Camplejohn RS. Improving the detection of p53 mutations in breast cancer by use of the FASAY, a functional assay. *J Mol Diagn* 2000, **2**(3), 139–144.
38. Chappuis PO, Estreicher A, Dieterich B, Bonnefoi H, Otter M, Sappino AP, *et al*. Prognostic significance of p53 mutation in breast cancer: frequent detection of non-missense mutations by yeast functional assay. *Int J Cancer* 1999, **84**(6), 587–593.
39. Fisher CJ, Gillett CE, Vojtesek B, Barnes DM, Millis RR. Problems with p53 immunohistochemical staining: the effect of fixation and variation in the methods of evaluation. *Br J Cancer* 1994, **69**(1), 26–31.
40. Kasper HU, Ebert M, Malfertheiner P, Roessner A, Kirkpatrick HK, Wolf HK. Expression of thrombospondin-1 in pancreatic carcinoma: correlation with microvessel density. *Virchows Arch* 2001, **438**(2), 116–120.
41. Straume O, Akslen LA. Expresson of vascular endothelial growth factor, its receptors (FLT-1, KDR) and TSP-1 related to microvessel density and patient outcome in vertical growth phase melanomas. *Am J Pathol* 2001, **159**(1), 223–235.
42. Gasparini G, Toi M, Biganzoli E, Dittadi R, Fanelli M, Morabito A, *et al*. Thrombospondin-1 and -2 in node-negative breast cancer: correlation with angiogenic factors, p53, cathepsin D, hormone receptors and prognosis. *Oncology* 2001, **60**(1), 72–80.
43. Dameron KM, Volpert OV, Tainsky MA, Bouck N. Control of angiogenesis in fibroblasts by p53 regulation of thrombospondin-1. *Science* 1994, **265**(5178), 1582–1584.
44. Iruela-Arispe ML, Lombardo M, Krutzsch HC, Lawler J, Roberts DD. Inhibition of angiogenesis by thrombospondin-1 is mediated by 2 independent regions within the type 1 repeats. *Circulation* 1999, **100**(13), 1423–1431.
45. Nor JE, Mitra RS, Sutorik MM, Mooney DJ, Castle VP, Polverini PJ. Thrombospondin-1 induces endothelial cell apoptosis and inhibits angiogenesis by activating the caspase death pathway. *J Vasc Res* 2000, **37**(3), 209–218.
46. Jimenez B, Volpert OV, Crawford SE, Febbraio M, Silverstein N, Bouck N. Signals leading to apoptosis-dependent inhibition of neovascularization by thrombospondin-1. *Nat Med* 2000, **6**(1), 41–48.
47. Taraboletti G, Morbidelli L, Donnini S, Parenti A, Granger HJ, Giavazzi R, *et al*. The heparin binding 25 kDa fragment of thrombospondin-1 promotes angiogenesis and modulates gelatinase and TIMP-2 production in endothelial cells. *Faseb J* 2000, **14**(12), 1674–1676.
48. Chandrasekaran L, He CZ, Al-Barazi H, Krutzsch HC, Iruela-Arispe ML, Roberts DD. Cell contact-dependent activation of alpha3beta1 integrin modulates endothelial cell responses to thrombospondin-1. *Mol Biol Cell* 2000, **11**(9), 2885–2900.