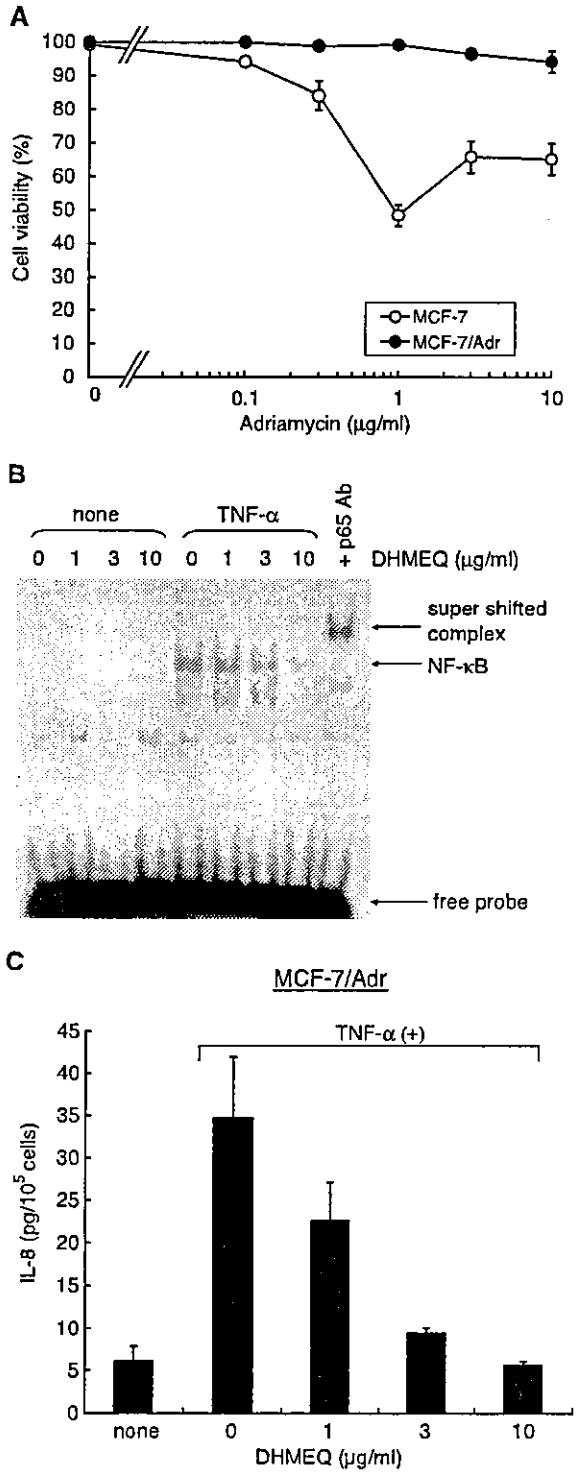
evaluated by staining with antibody M30, which reacts with caspase-cleaved neoepitope of cytokeratin 18. The expression of apoptosis in the DHMEQ treatment group was higher than that in the control group (Fig. 6*C*, *bottom*).



Inhibition of TNF-α-Induced NF-κB Activation by DHMEQ in MCF-7/Adr Cells. MCF-7/Adr cells highly expressing MDR1 were resistant to Adriamycin compared with the parental cells (Fig. 7*A*), but there was no constitutive activation of NF-κB in MCF-7/Adr cells (Fig. 7*B*). DHMEQ almost completely inhibited TNF-α-induced activation of NF-κB in these cells. The expression level of IL-8 from MCF-7/Adr cells was not different from that of parental cells (Fig. 7*C*). DHMEQ almost completely inhibited TNF-α-induced secretion of IL-8 from MCF-7/Adr cells.

DISCUSSION

DHMEQ inhibited NF-κB activation and secretion of inflammatory cytokines such as IL-6 and IL-8 in breast carcinoma cells. Continuous elevation of IL-6 levels was reported as a factor indicative of a poor prognosis in heavily pretreated patients with recurrent breast cancer (23). In MCF-7 cells, overexpression of human manganese superoxide dismutase, which may inhibit NF-κB, suppresses tumor growth both *in vitro* and *in vivo* by down-regulating genes responsible for tumor malignant phenotype including IL-6 (24). Elevated expression of IL-8 was reported to mediate invasiveness, angiogenesis, and metastasis of breast cancer cells in association with ER status and to influence prognosis (25, 26). ER-positive cells often expressed low levels of IL-8, whereas ER-negative ones often expressed high levels of IL-8 (27, 28). In our experiment, ER-negative MDA-MB-231 cells constitutively expressed a high level of IL-8 (Fig. 5*C*) compared with ER-positive MCF-7 cells (Fig. 5*E*). TNF-α treatment enhanced the levels in both cell lines. DHMEQ inhibited TNF-α-stimulated IL-8 secretion in both kinds of cells (Fig. 5*D-E*). Thus it is possible that the suppression of MDA-MB-231 and MCF-7 tumors *in vivo* by DHMEQ may be partly due to the inhibition of cytokine secretions.

NF-κB has also been shown to up-regulate the expression of several proangiogenic genes, directly or indirectly, including urokinase-type plasminogen activator, matrix metalloproteinase 9, and vascular endothelial growth factor. Therefore, the suppression of NF-κB could also be an antiangiogenic treatment for cancer. Copper deficiency, which inhibits NF-κB, contributes to a global inhibition of NF-κB-mediated transcription of proangiogenic factors such as vascular endothelial growth factor, fibroblast growth factor-2, IL-1, IL-6, and IL-8 in breast cancer (29). DHMEQ is likely to decrease these proangiogenic factors.

Fig. 7 Inhibition of TNF-α-induced NF-κB activation and cytokine secretion by DHMEQ in multidrug-resistant MCF-7/Adr cells. *A*, drug resistance in MCF-7/Adr cells. MCF-7 cells (○) and MCF-7/Adr cells (●) were treated with various concentrations of Adriamycin for 48 hours. Then, cell viability was assessed by the trypan blue dye exclusion assay. *B*, inhibition of NF-κB activation. MCF-7/Adr cells were treated with the indicated concentrations of DHMEQ for 2 hours, and then 20 ng/mL TNF-α was added and incubation continued for 30 minutes. The nuclear extract was assayed by EMSA. *C*, inhibition of TNF-α-induced IL-8 secretion in MCF-7/Adr cells. The cells were treated with the indicated concentrations of DHMEQ for 2 hours, and then stimulated with 20 ng/mL TNF-α for 3 hours. IL-8 production was assessed by ELISA. The values are mean ± SD of quadruplicate determinations.

LT-β was discovered as a glycoprotein receptor that binds to LT-α/TNF-β at the cell-surface membrane (30). When LT forms a homotrimer, LT-α_3, it binds to TNF-α receptors 1 and 2; but when LT forms a LT-$\alpha_1\beta_2$ heterotrimer, it binds to the LT-β receptor. Using MCF-7 cells, we found that DHMEQ inhibits both TNF-α receptor 1 and LT-β-mediated activations of NF-κB (Fig. 4*A-C*).

Chemoresistance toward Adriamycin, which is a key drug for the treatment of breast cancer, is a serious problem. The expression of P-glycoprotein encoded by the multidrug resistance (MDR1) gene is often elevated by clinical use of Adriamycin. MCF-7/Adr cells are the typical resistant cells expressing large amounts of MDR1. We found that DHMEQ was equally effective for the inhibition of NF-κB and cytokine secretion in MCF-7/Adr cells. Therefore, DHMEQ should not be a substrate of MDR1.

Thus, DHMEQ inhibited activation of NF-κB and cytokine secretion in breast carcinoma cells including ER-negative and ER-positive ones. It was also effective in MDR1-expressing breast carcinoma cells. DHMEQ had an excellent therapeutic potential in both antiangiogenesis and apoptosis induction in xenograft tumors of breast carcinomas. Previously, it was shown to inhibit animal models of prostate carcinoma (18), rheumatoid arthritis (16), and renal inflammation (31) without any toxicity. DHMEQ may be a candidate of chemotherapeutic agent for breast carcinomas targeting NF-κB.

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Association between intratumoral free and total VEGF, soluble VEGFR-1, VEGFR-2 and prognosis in breast cancer

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Vascular endothelial growth factor (VEGF) receptors consist of three cell-membrane type receptors (VEGFR-1, VEGFR-2 and VEGFR-3), and soluble form of VEGFR-1 (sVEGFR-1), an intrinsic negative counterpart of the VEGF. In this study, we measured intratumoral protein levels of free and total VEGF, VEGFR-2 and sVEGFR-1 from 202 primary breast cancer tissues and examined their prognostic values. A significant inverse correlation was found between free or total VEGF and oestrogen receptor (ER) status ($P = 0.042$ and 0.032, respectively). A univariate analysis showed that low sVEGFR-1 and high total VEGF were significantly associated with poor prognosis in disease-free survival (DFS) and overall survival (OS). The ratio of sVEGFR-1 to total VEGF was a strong prognostic indicator (DFS: $P = 0.008$; OS: $P = 0.0002$). A multivariate analysis confirmed the independent prognostic values of total VEGF and the ratio of sVEGFR-1 to total VEGF. In subgroup analysis, total VEGF was a significant prognostic indicator for ER-positive tumours but not for ER-negative tumours, whereas sVEGFR-1 was significant for ER-negative tumours but not for ER-positive tumours. In conclusion, the intratumoral sVEGFR-1 level, VEGF level and the ratio of sVEGFR-1 to total VEGF are potent prognostic indicators of primary breast cancer, and might be relevant to ER status.
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Vascular endothelial growth factor (VEGF) and its receptors are essential for neovascularisation in cancer. Numerous studies have indicated that intratumoral VEGF expression is significantly correlated with microvessel density and poor prognosis in a variety of human solid cancers including breast cancer, brain tumours, head and neck cancer and gastrointestinal cancer (Toi *et al*, 2001; Ferrara *et al*, 2003). The prognostic value of VEGF has been confirmed not only in immunohistochemical studies but also in other studies using enzyme-linked immunosorbent assay (ELISA) and Northern blotting. In most clinical studies that examined the prognostic value of VEGF in primary breast cancer, intratumoral VEGF expression was a significant marker of poor prognosis in both node-negative and node-positive subgroups (Gasparini, 2000). Thus, these studies concluded that intratumoral VEGF status is an independent prognostic indicator of primary breast cancer.

VEGF binds to two types of cell-membrane receptors, the VEGF receptor (VEGFR)-1 and VEGFR-2 located in the endothelium, and stimulates endothelial migration, proliferation, permeability and survival (Ferrara and Alitalo 1999; Shibuya, 2001). In addition to these two receptors, a soluble form of VEGFR-1 (sVEGFR-1), a naturally occurring and alternatively spliced variant of sVEGFR-1, functions as a high-affinity receptor of VEGF (Kendall and Thomas, 1993; Kendall *et al*, 1996). Since sVEGFR-1 is a secretory protein, it is an intrinsic negative counterpart of VEGF signalling. Recombinant sVEGFR1 binds to all isoforms of VEGF and inhibits VEGF-induced endothelial cell proliferation. Gene therapies involving sVEGFR-1 significantly suppress tumour growth in animal experimental models (Goldman *et al*, 1998; Takayama *et al*, 2000; Mahasreshti *et al*, 2001; Hoshida *et al*, 2002; Sako *et al*, 2004). Kendall *et al* (1996) found that sVEGFR1 is abundant in all identified VEGFR-1 cDNAs from human primary endothelial cells. In our preliminary study using primary breast cancer tissues, sVEGFR1 was frequently coexpressed with VEGF, and the intratumoral balance between sVEGFR1 and VEGF levels showed a significant relationship with survival (Toi *et al*, 2002). According to a recent report on brain tumours, the ratio of sVEGFR-1 to VEGF is significantly decreased in glioblastomas compared with astrocytomas, which indicates the importance of sVEGFR-1 expression in brain tumour growth (Lamszus *et al*, 2003). It is therefore crucial to investigate the relationship between VEGF and its receptors including sVEGFR-1. Previous studies have indicated that intratumoral sVEGFR-1 levels are frequently elevated in

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human tumour tissues, although the precise upregulation mechanism involving sVEGFR-1 in cancer is largely unknown.

Recently, we have developed a more sensitive sVEGFR-1 ELISA system in addition to new methodologies that permit the separate measurement of free VEGF, which is unbound to the receptors, and total VEGF, which includes both bound and unbound molecules to receptors. In this study, we measured total and free VEGF, sVEGFR-1 and VEGFR-2, as well as Her-2/neu and thymidine phosphorylase (TP) levels in breast tumour cytosols quantitatively and then evaluated those prognostic values. The information from this study is useful not only for assessing the prognostic value of these markers but also for considering the clinical implications of future anti-VEGF therapies paradigm.

MATERIALS AND METHODS

Patient population

We randomly selected tissues from 202 patients with operable primary breast cancer who underwent modified radical or partial mastectomy with full dissection of their axillary lymph nodes at the Tokyo Metropolitan Komagome Hospital from 1996 to 1999 with an average follow-up period of 64.0 months and a range of 1.3–93.2 months. Representative samples of the tumour specimens were immediately frozen in liquid nitrogen after surgical resection and stored at −80°C until preparation for ELISA. Pathological examinations were performed on formalin-fixed, paraffin-embedded specimens. The main characteristics of the patients and adjuvant hormone and chemotherapy details are described in Table 1. All patients signed an informed consent according to a protocol approved by the ethics committee of the hospital.

Table 1 Patients characteristics

	Number of patients (%)
Patients enrolled	202
Median age (years)	55, range 30–86
Menopausal status	
Pre	86 (42.6)
Post	116 (57.4)
Tumour size (cm)	
<2	26 (12.9)
2–5	125 (61.9)
>5	51 (25.2)
Nodal involvement	
−	94 (46.5)
+	108 (53.5)
ER	
Positive	110 (54.5)
Negative	92 (45.5)
PgR	
Positive	105 (52.0)
Negative	97 (48.0)
Hormonal receptor	
ER+ and PR+	79 (39.1)
ER+ or PR+	56 (27.7)
ER− and PR−	67 (33.2)
Nuclear grade	
1	39 (19.3)
2	99 (49.0)
3	64 (31.7)
Recurrence	
+	57 (28.2)
−	145 (71.8)
Adjuvant therapy	
CAF (CEF)	54 (26.7)
CMF (CF)	18 (8.9)
FU derivatives	38 (18.8)
Tamoxifen	119 (58.9)
LH-RH	10 (5.0)

ER = oestrogen receptor; PgR = progesterone receptor; CAF (CEF) = cyclophosphamide, adriamycin or epirubicin and 5-FU; CMF (CF) = cyclophosphamide, methotrexate and 5-FU.

Adjuvant therapy and patient follow-up Indications for and the schedule of adjuvant treatment were decided based on the patient characteristics including axillary nodal involvement (n), tumour size (T), age and oestrogen receptor (ER). Polychemotherapy including six cycles of CA(E)F (cyclophosphamide, adriamycin/epirubicin and 5-fluorouracil (5-FU)) was given to node-positive patients under the age of 60 years, and FU derivatives were given to the remaining node-positive and high-risk node-negative patients. Tamoxifen was given to hormone receptor-positive patients without a history of thrombosis or liver dysfunction for 5 years and additional LH-RH agonist therapy was undertaken for 2 years for premenopausal cases. The patients received radiation to the remaining breast if partial mastectomy was performed, and to the chest wall and draining lymph nodes if more than four lymph nodes were involved. Post-treatment surveillance was carried out according to general practice for breast cancer patients at our institute. Briefly, for the first 5 years physical examinations, haematology and blood chemistry analyses were performed every 3 months, chest X-rays were taken every 6 months and mammography was performed annually. Thereafter, physical, blood and chest X-ray examinations were performed every 6–12 months, and annual mammography was continued. If tumour relapse was suspected, the patient underwent intensive work-up including chest/abdominal computed tomography scans, isotopic bone scans, bone radiography or histological examination. Survival analysis was performed on 186 cases excluding six patients with ductal carcinoma *in situ* (DCIS) and 10 patients who did not show up for the follow-up. The outcomes examined included overall survival (OS) and disease-free survival (DFS), which were calculated from the date of surgery. Overall survival was calculated from the date of surgery to last contact for living patients. Disease-free survival was defined as the period from the date of surgery to the confirmed tumour relapse date for relapsed patients and from the date of surgery to the date of the last follow-up for disease-free patients.

Histopathologic analysis

Representative sections from all primary tumours were reviewed and analysed by pathologists. The special morphologic features examined included grade, lymph vessel/blood vessel involvement and the number of lymph nodes involved.

Sample preparation Breast tumour tissue samples were treated with two different types of lysis buffer. For the measurement of sVEGFR-1 and TP protein, tissue samples were homogenised in a solution of 10 mM Tris-HCl buffer (pH 7.4) containing 15 mM NaCl, 1.5 mM $MgCl_2$, 50 μM potassium phosphate and a protease-inhibitor cocktail. For all other ELISA measurements, samples were individually homogenised in a 10-fold volume of RIPA buffer (0.1% SDS, 1% Tween 20, 0.5% Na-deoxycholate, protease-inhibitor cocktail in phosphate-buffered saline, pH 7.4) and then centrifuged at 14 000 g for 20 min. The supernatants were then stored at −80°C until use. A portion of each supernatant was used for protein concentration measurement according to standard protocols (BCA assay, Pierce, Rockford, IL, USA).

Enzyme-linked immunosorbent assay

Total VEGF protein concentrations in the tumour cytosols were measured using VEGF ELISA kits (R&D Systems, Minneapolis, MN, USA). The measurements were conducted according to the

methods recommended by the manufacturer. The minimal detection limit for total VEGF was 31 pg ml^{-1}.

A receptor–ligand detection assay was applied to detect free bioactive VEGF following the basic protocol for total VEGF ELISA, except that plates were coated with 0.5 μg ml^{-1} sVEGFR-1 (D1–D6) produced in insect cells (Hornig *et al*, 1999). This ensured that no VEGFR-1 complex forms were recognised. For detection, biotinylated anti-VEGF antibody (R&D Systems, Minneapolis, MN, USA) was used. The minimal detection limit for free VEGF was 20 pg ml^{-1}.

Enzyme-linked immunosorbent assay for sVEGFR-1 was performed as previously reported with modifications to improve sensitivity (Toi *et al*, 2002). A human sVEGFR-1 ELISA kit (Bender MedSystems, Vienna, Austria) was used according to the manufacturer's protocol. The minimum detection limit was 100 pg ml^{-1}.

The VEGFR-2 protein concentration in tumour lysates was measured using VEGFR-2 ELISA kits (R&D Systems, Minneapolis, MN, USA). The measurements were conducted according to the methods recommended by the manufacturer. The minimal detection limit for VEGFR-2 was 78 pg ml^{-1}.

Her-2/neu was determined using a Her-2/neu (c-erbB-2) sandwich enzyme immunoassay (Oncogene Science, Cambridge, MA, USA), which employs a mouse monoclonal antibody for capture and a different biotinylated mouse monoclonal antibody for the detection of human neu protein. The capture and detector reagents specifically bind to the extracellular domain of the neu protein. The minimal detection limit for Her-2/neu was 24 pg ml^{-1}.

Thymidine phosphorylase levels were also determined by a colorimetric ELISA. This sandwich immunoassay used two anti-human TP monoclonal antibodies (Nippon Roche Research Center, Kamakura, Japan; 104B and 232-2). The minimal detectable concentration was 1.25 ng ml^{-1}.

Levels of ER and progesterone receptor (PgR) were determined using enzyme immunoassay systems from the Otsuka Assay Institute (Tokushima, Japan) as previously reported. The cutoff value of enzyme immunoassay for ER and PgR was 10 fmol mg^{-1} protein.

All protein level measurements made by ELISA were performed in duplicate.

Statistical methods

The correlation between two factors was evaluated using the Spearman's correlation coefficient by rank and unpaired groups were compared using the Student's *t*-test. Univariate and multivariate Cox regression analyses were carried out to assess potential prognostic indicators of DFS and OS. These features included ER and PgR status, tumour grade (low *vs* intermediate and high grade), tumour size (5 *vs* >5 cm), axillary lymph node involvement (positive *vs* negative), lymph vessel involvement (positive *vs* negative), blood vessel involvement (positive *vs* negative), total VEGF protein concentration (<mean *vs* >mean), free VEGF protein concentration (<mean *vs* >mean), sVEGFR-1 protein concentration (<0.435 *vs* >0.435 ng mg^{-1} protein), VEGFR-2 protein concentration (mean *vs* >mean), Her-2/neu protein concentration (<mean *vs* >mean), TP protein concentration (mean *vs* >mean) and sVEGFR-1/total VEGF (<0.5 *vs* $\geqslant$0.5). All clinical and biological parameters regardless of whether they were statistically significant as seen by the univariate analysis were included in the multivariate analysis. Variables that exhibited statistically significant effects were then retained and the others were dropped.

Multivariate analysis resulted in a final model of five prognostic variables for DFS and four prognostic variables for OS. Models were then generated based on the presence or absence of these variables and constructed to assess the relative risk for relapse and death.

Standard Kaplan–Meier and Cox regression methods were applied for survival analysis using the StatView statistical software Version 5.0 (SAS Institute, Cary, NC, USA). All significance testing was two-sided, where log-rank statistics and Wald statistics were used for univariate and multivariate analysis, respectively. Differences for $P<0.05$ were considered to be statistically significant. The last follow-up date was 31st March 2004.

RESULTS

Patient characteristics

The patient characteristics are listed in Table 1. The median age at diagnosis was 55 years with a range of 30–86 years. Five patients who presented with DCIS were excluded from the survival analysis. A total of 110 (54%) underwent adjuvant chemotherapy, and 64% of the patients with positive receptor underwent adjuvant hormonal therapy. In all, 66% of the tumours were ER positive and/or PgR positive, and 81% were intermediate or high-grade tumours.

The protein concentrations of total VEGF, free VEGF, sVEGFR-1, VEGFR-2, TP and Her-2/neu in breast tumour tissue extracts determined by ELISA are listed in Table 2. The correlations between each factor and clinico-pathological parameters were analysed. Total and free VEGF levels were significantly higher in ER-negative tumours, and free VEGF levels were also higher in PgR-negative tumours. Soluble VEGFR-1 levels were higher in PgR-negative tumours, and VEGFR-2 showed no statistically significant correlation with any of the clinico-pathological parameters. Her-2/neu was associated with a larger tumour size, ER negativity and high nuclear grade ($P=0.01$, 0.04 and 0.03, respectively). There was a significant correlation of the protein levels between total VEGF and free VEGF ($P<0.001$, $\rho=0.905$), total VEGF and sVEGFR-1 ($P<0.001$, $\rho=0.278$), free VEGF and sVEGFR-1 ($P<0.001$, $\rho=0.251$), sVEGFR-1 and VEGFR-2 ($P=0.008$, $\rho=0.190$), total VEGF and Her-2/neu ($P=0.029$, $\rho=0.157$), free VEGF and Her-2/neu ($P=0.04$, $\rho=0.145$), total VEGF and TP ($P=0.004$, $\rho=0.207$), free VEGF and TP ($P=0.017$, $\rho=0.171$) and sVEGFR-1 and TP ($P<0.001$, $\rho=0.270$), but none was seen between VEGF and VEGFR-2 by Spearman's rank correlation test (data not shown).

To assess the prognostic value of sVEGFR-1 and the ratio of sVEGFR-1 to total VEGF, we determined the cutoff level according to a stepwise method that gives the optimal separation between a low and high risk of relapse as previously described (Toi *et al*, 2002). The cutoff value for sVEGFR-1 was 0.435 ng mg^{-1} protein, which identified 15.6% of the patients enrolled in the survival analysis as having low sVEGFR-1. For total and free VEGF, VEGFR-2, TP and Her-2/neu, the cutoff values were determined as their respective mean values.

According to the combination analysis, the ratio of sVEGFR-1 to total VEGF concentration (S/V ratio) and its prognostic value were assessed using a similar stepwise separation method as described above. Tumours with an associated S/V ratio of 3.0 were considered to be borderline for achieving a prognostic value for survival analysis. A value of 0.5 was decided on as the cutoff value, with 8.6% of the patients having a low S/V ratio with the most unfavourable prognosis (Table 3).

For univariate analyses, patients with low-grade tumours ($P=0.002$), tumours less than 5 cm in size ($P=0.0001$), no lymph node involvement ($P=0.0001$), less vessel involvement ($P=0.01$–0.001), low total VEGF level ($P=0.002$), low free VEGF level ($P=0.047$), high sVEGFR-1 level ($P=0.04$) and a high S/V ratio ($P=0.008$) experienced favourable DFS (Table 3). Overall survival was favourable for patients with negative PgR status ($P=0.017$), low-grade tumours ($P=0.011$), tumours less than 5 cm in size ($P=0.0002$), no lymph node involvement ($P=0.0005$), less vessel

Table 2 Quantitation of total and free VEGF, sVEGFR-1, VEGFR-2, Her-2/neu and TP proteins in primary breast cancer tumour cytosol

		Total VEGF			Free VEGF			sVEGFR-1			VEGFR-2			TP			Her-2/neu		
	No.	Mean	95% CI	P	Mean	95% CI	P	Mean	95% CI	P	Mean	95% CI	P	Mean	95% CI	P	Mean	95% CI	P
Patients enrolled	202	0.532	0.432–0.632		0.135	0.118–0.152		0.949	0.849–1.048		0.105	0.098–0.112		194.9	181.1–209.9		13.057	10.147–15.867	
Menopause																			
Pre	86	0.534	0.420–0.644	NS	0.132	0.106–0.170	NS	0.896	0.740–1.053	NS	0.102	0.087–0.131	NS	194.2	177.9–219.1	NS	15.265	10.189–20.340	NS
Post	116	0.530	0.416–0.644		0.141	0.113–0.156		0.994	0.858–1.130		0.108	0.098–0.123		195.3	172.8–213.4		11.244	8.005–14.483	
Tumour size																			
T1	26	0.532	0.420–0.644	NS	0.102	0.075–0.128	NS	0.917	0.737–1.97	NS	0.103	0.089–0.117	NS	196.7	166.3–227.1	NS	11.916	5.163–18.669	
T2	125	0.536	0.425–0.647		0.141	0.115–0.168		0.962	0.823–1.097		0.107	0.095–0.119		198.6	178.7–218.4		10.785	7.563–14.007	
T3 or more	51	0.520	0.356–0.684		0.136	0.117–0.155		0.956	0.714–1.198		0.105	0.085–0.125		187.0	155.6–218.5		19.290	12.006–26.575	0.01[a]
Nodal status																			
n−	94	0.550	0.462–0.638	NS	0.139	0.116–0.163	NS	0.954	0.852–1.055	NS	0.106	0.096–0.116	NS	201.9	188.9–214.9	NS	11.983	8.285–15.682	NS
n+	108	0.516	0.392–0.640		0.133	0.102–0.163		0.899	0.751–1.047		0.104	0.091–0.117		187.6	166.5–208.5		13.860	9.568–18.152	
Hormonal receptor																			
ER+	120	0.458	0.401–0.515	0.042	0.119	0.098–0.140	0.032	0.905	0.784–1.027	NS	0.109	0.098–0.121	NS	201.0	183.6–218.4	NS	10.924	7.985–13.864	0.048
ER−	82	0.621	0.464–0.778		0.159	0.092–0.227		1.031	0.849–1.213		0.102	0.087–0.117		186.9	161.5–212.3		16.117	10.473–21.761	
PgR+	105	0.450	0.342–0.559	0.055	0.112	0.087–0.138	0.012	0.81	0.695–0.926	0.011	0.103	0.094–0.112	NS	190.2	181.0–210.5	NS	12.170	7.957–16.384	NS
PgR−	97	0.600	0.491–0.710		0.160	0.133–0.186		1.094	0.933–1.254		0.110	0.098–0.122		200.3	179.5–221.1		13.817	9.870–17.765	
Recurrence																			
+	57	0.658	0.516–0.800	NS	0.163	0.129–0.197	NS	0.942	0.741–1.144	NS	0.103	0.082–0.123	NS	203.6	169.8–237.7	NS	13.612	10.285–16.939	NS
−	145	0.482	0.3801–0.584		0.126	0.104–0.148		0.941	0.825–1.057		0.107	0.098–0.116		192.8	177.1–208.6		11.491	5.617–17.366	
Nuclear grade																			
1	39	0.531	0.450–0.612	NS	0.104	0069–0.138	NS	0.868	0.849–1.048	NS	0.110	0.091–0.129	NS	186.9	168.4–205.4	NS	12.965	6.758–19.172	
2	99	0.503	0.400–0.606		0.141	0.112–0.170		0.917	0.772–1.062		0.099	0.089–0.109		200.8	180.9–220.7		10.323	6.969–13.678	
3	64	0.578	0.468–0.688		0.148	0.116–0.180		1.075	0.864–1.286		0.110	0.095–0.125		205.6	178.4–232.8		16.947	10.680–23.214	0.032[b]

Intratumoral total and free VEGF, sVEGFR-1, VEGFR-2, TP, Her-2/neu, ER and PR protein levels were measured by quantitative ELISA and enzyme immunoassay (see 'Materials and Methods'). The results reflect the mean values, 95% CI and *P*-value. Levels of ER and PgR more than 10 fmol mg^{-1} total protein were considered positive (+) and less than 5 fmol mg^{-1} total protein negative (−). The correlations between each biological factor and clinico-pathological parameters were analysed using Student's *t*-test. Differences at $P<0.05$ were considered to be statistically significant. NS = not significant. [a]Statistically significant between T2 and T3. [b]Statistically significant between nuclear grade 2 and 3.

Table 3 Univariate analysis of clinico-pathological and tumour biologic factors for DFS and OS

		DFS			OS		
Parameter	***n* (total 186)**	***P***	**Hazard ratio**	**95% CI**	***P***	**Hazard ratio**	**95% CI**
Clinical features							
ER							
Positive	111	0.503	0.835	0.493–1.415	0.098	0.604	0.332–1.099
Negative	75	Baseline			Baseline		
PgR							
Positive	92	0.051	0.584	0.340–1.003	0.017	0.461	0.243–0.872
Negative	94	Baseline			Baseline		
Nuclear grade							
1	33	0.002	0.111	0.026–0.466	0.011	0.075	0.010–0.556
2 and 3	153	Baseline			Baseline		
Tumour size (cm)							
1–5	146	0.0001	0.224	0.131–0.384	0.0002	0.313	0.170–0.579
5 or more	42	Baseline			Baseline		
Nodal involvement							
n+	104	Baseline			Baseline		
n−	84	0.0001	0.207	0.104–0.410	0.0005	0.271	0.130–0.565
Lymph vessel (ly) involvement							
ly positive	140	Baseline			Baseline		
ly negative	46	0.001	0.107	0.026–0.438	0.008	0.07	0.010–0.512
Blood vessel (v) involvement							
v positive	131	Baseline			Baseline		
v negative	55	0.016	0.396	0.187–0.842	0.05	0.421	0.177–1.002
Biological features							
Total VEGF							
High (>0.532 ng mg^{-1} protein)	64	0.002	2.231	1.306–3.811	0.006	2.278	1.233–4.206
Low (<0.532 ng mg^{-1} protein)	122	Baseline			Baseline		
Free VEGF							
High (>0.135 ng mg^{-1} protein)	71	0.047	1.72	0.999–2.962	0.204	1.488	0.803–2.758
Low (<0.135 ng mg^{-1} protein)	115	Baseline			Baseline		
SVEGFR-1							
High (>0.435 ng mg^{-1} protein)	29	0.040	0.526	0.282–0.983	0.05	0.527	0.258–1.075
Low (<0.435 ng mg^{-1} protein)	157	Baseline			Baseline		
VEGFR-2							
High (>0.105 ng mg^{-1} protein)	119	0.316	0.762	0.447–1.299	0.883	1.047	0.563–1.947
Low (<0.105 ng mg^{-1} protein)	67	Baseline			Baseline		
Her-2/neu							
High (>13.5 ng mg^{-1} protein)	41	Baseline			Baseline		
Low (<13.5 ng mg^{-1} protein)	145	0.362	1.394	0.682–2.848	0.753	0.889	0.426–1.854
TP							
High (>194.9 ng mg^{-1} protein)	97	0.785	0.928	0.541–1.590	0.955	0.983	0.533–1.813
Low (<194.9 ng mg^{-1} protein)	89	Baseline			Baseline		
S/V ratio							
High (≥0.5)	170	0.008	0.368	0.186–0.731	0.0002	0.267	0.127–0.561
Low (<0.5)	16	Baseline			Baseline		

Prognostic parameters evaluated included ER and PR status (< 10 fmol mg^{-1} protein *vs* more than 10 fmol mg^{-1} protein), primary tumour size, axillary lymph node involvement, total VEGF level, free VEGF level, sVEGFR-1 level, VEGFR-2 level, Her-2/neu protein level, TP level and S/V ratio (sVEGFR-1/total VEGF ratio). For total and free VEGF, VEGFR-2 and Her-2 protein levels, cutoff values were determined as mean values. For sVEGFR-1 and S/V ratio, cutoff values were determined according to a stepwise method (see Results). The median follow-up was 64 months. Survival analysis was performed on 186 cases excluding six patients with ductal carcinoma *in situ* and 10 patients who did not show up for the follow-up. The prognostic significance was assessed using the log-rank test. All *P*-values are two-sided. Hazard ratio indicated Cox model hazard ratio.

involvement ($P=0.05-0.008$), low total VEGF level ($P=0.006$), high sVEGFR-1 level ($P=0.05$) and a high S/V ratio ($P=0.0002$). Figure 1 shows DFS curves of the tumour-related prognostic features total VEGF and sVEGFR-1. Oestrogen receptor status and VEGFR-2, Her-2/neu and TP level did not have a statistically significant effect on patient outcome in the univariate analyses.

When we assessed the prognostic value of angiogenesis-related factors in the subgroups divided by ER status, total VEGF was a significant prognostic factor for ER-positive group ($P=0.0003$) and not for ER-negative group ($P=0.120$) (Table 4 and Figure 1). In contrast, within the ER-negative group, sVEGFR-1 and the S/V ratio were found to be strong prognostic indicators ($P=0.001$ and 0.0001, respectively), but this was not the case for the ER-positive group. In particular, all patients with ER-negative and low S/V ratio tumours relapsed within 4 years after surgery (Figure 1). There was also a statistically significant benefit for OS with high sVEGFR-1 or a high S/V ratio for the ER-negative group ($P=0.03$ and 0.0002, respectively), but not for the ER-positive group. Figure 1 shows the DFS curves for total VEGF, sVEGFR-1 and the S/V ratio for the ER-positive population ($n=111$) and ER-negative subpopulation ($n=75$). The results of other subgroup analyses divided by total VEGF status, TP status and Her-2/neu status are summarised in Table 4.

All tumour- and clinico-pathological-related parameters regardless of whether they were statistically significant as seen by univariate analysis were included in the multivariate analysis. Variables showing statistically significant effects were retained and the others were dropped. The resulting multivariate analysis

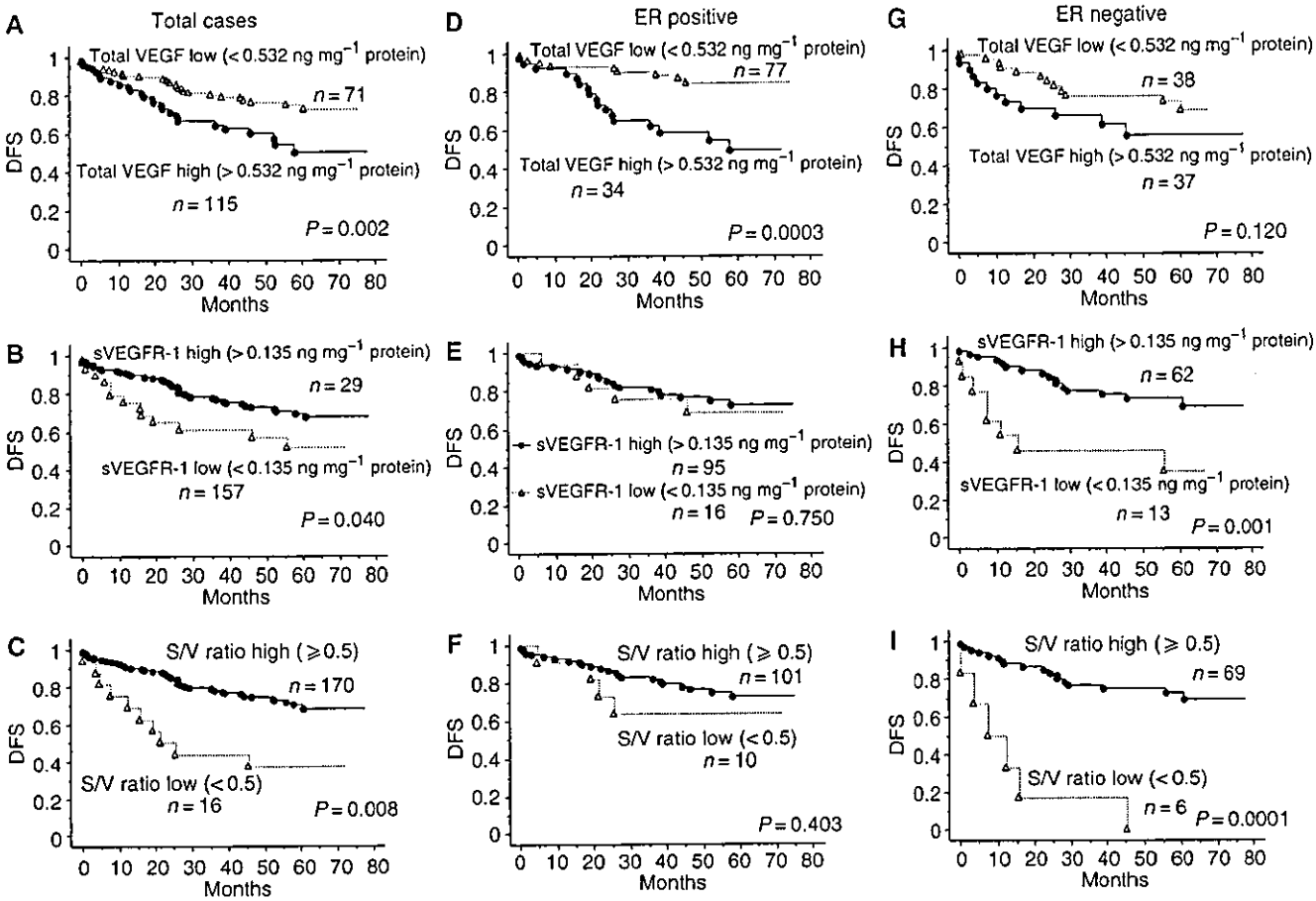

Figure 1 Kaplan–Meier curves for DFS in patients with primary breast cancer by biological markers. Kaplan–Meier curves for DFS in patients with primary breast cancer by biological markers. Disease free survival in the total cases ($n = 186$) by VEGF level (**A**), sVEGFR-1 level (**B**) and sVEGFR-1/totalVEGF ratio (S/V ratio; **C**). Disease free survival in the ER-positive cases ($n = 111$) by VEGF level (**D**), sVEGFR-1 level (**E**) and S/V ratio (**F**): Disease free survival in the ER-negative cases ($n = 75$) by VEGF level (**G**), sVEGFR-1 level (**H**) and S/V ratio (**I**). Levels of VEGF protein more than 0.532 ng mg^{-1} total protein were considered high (solid line) and less than 0.532 ng mg^{-1} total protein negative (dotted line) in (**A**, **D** and **G**). Soluble VEGFR-1 protein levels more than 0.135 ng mg^{-1} total protein were considered high (solid line) and less than 0.135 ng mg^{-1} total protein negative (dotted line) in (**B**, **E** and **H**). S/V ratio more than 0.5 was considered high (solid line) and less than 0.5 negative (dotted line) in (**C**, **F** and **I**). (**A**) The hazard ratio (HR) = 2.23 (95% confidence interval (CI) = 1.31–3.81, $P = 0.002$ using the log-rank test) in favour of total VEGF-low group. (**B**) HR = 0.526 (95% CI = 0.28–0.98, $P = 0.04$ using the log-rank test) in favour of total sVEGFR-1-high group. (**C**) HR = 0.368 (95% CI = 0.19–0.73, $P = 0.008$ using the log-rank test) in favour of S/V ratio-high group. (**D**) In ER-positive subgroup, HR = 3.800 (95% CI = 1.74–8.31, $P = 0.0003$ using the log-rank test) in favour of total VEGF-low group. (**H**) In ER-negative subgroup, HR = 0.269 (95% CI = 0.11–0.53, $P = 0.001$ using the log-rank test) in favour of sVEGFR-1-high group. (**I**) In ER-negative subgroup, HR = 0.114 (95% CI = 0.04–0.30, $P = 0.0001$ using the log-rank test) in favour of sVEGFR-1-high group.

revealed that DFS and OS were improved in patients with low total VEGF ($P < 0.001$ and $P = 0.043$), a high S/V ratio ($P = 0.002$ and 0.003), pathological low grade ($P = 0.015$ and 0.034), tumour less than 5 cm in size ($P = 0.002$ and 0.038) and negative nodal involvement ($P = 0.001$ and 0.002, respectively) (Table 5). Soluble VEGFR-1 alone did not result in improved DFS or OS.

DISCUSSION

Soluble VEGFR1 levels are frequently elevated in human breast cancer tissues. Out of 202 tumours, 155 contained higher concentrations of sVEGFR-1 than those of total VEGF. A recent study of the relationship between circulating sVEGFR-1 levels and preeclampsia reported that increased sVEGFR-1 is significantly associated with the development of preeclampsia, suggesting that immunodetectable sVEGFR-1 is biologically active (Levine *et al*, 2004). Regarding malignancies such as brain tumours or leukaemia, it was also documented that intratumoral or plasma sVEGFR-1 level is related to tumour phenotype or prognosis, suggesting that sVEGFR-1 plays a significant biological role not only during development or pregnancy but also in neoplasms (Lamszus *et al*, 2003; Hu *et al*, 2004).

Since simultaneous measurement of sVEGFR-1 and VEGFR-1 in the same sample is technically difficult, we were not able to compare the concentrations of sVEGFR-1 with those of VEGFR-1 directly. Nevertheless, as a preliminary study, we examined sVEGFR-1 and VEGFR-1 expressions in human umbilical vein endothelial cells (HUVECs) and in primary breast tumour tissues by Western blot using different types of lysis buffers with or without detergent. Using HUVECs as a positive control for both VEGFR-1 and sVEGFR-1, we confirmed that in the protein extract prepared without detergent, only sVEGFR-1 was detectable, and in contrast, in the protein extract prepared with detergent such as with RIPA buffer, both VEGFR-1 and sVEGFR-1 were detectable. We speculated that VEGFR-1 exists on a cell membrane, and the membrane fraction will not be lysed in a buffer without detergent. Then we examined expression of sVEGFR-1 and VEGFR-1 by Western blot analysis in 15 randomly selected primary breast cancer tissues prepared with different lysis buffers: 11 tumours had

Table 4 Univariate subgroup analysis of tumour biologic factors for DFS and OS

										P-values												
	Total case (186 cases) 54 relapse		ER+ cases (111 cases) 27 relapse		ER− cases (75 cases) 27 relapse		PgR+ cases (92 cases) 20 relapse		PgR− cases (94 cases) 34 relapse		n+ cases (101 cases) 44 relapse		n− cases (85 cases) 10 relapse		TP-high cases (97 cases) 27 relapse		TP-low cases (89 cases) 27 relapse		Her-2/neu-high cases (41 cases) 9 relapse		Her-2/neu-low cases (145 cases) 45 relapse	
	DFS	OS	DFS	OS	DFS	OS	DFS	OS	DFS	OS	DFS	OS	DFS	OS	DFS	OS	DFS	OS	DFS	OS	DFS	OS
Total VEGF	0.002	0.006	0.0003	0.003	0.120	0.091	0.038	0.114	0.058	0.062	0.012	0.050	0.002	0.006	0.001	0.002	0.242	0.479	0.341	0.575	0.002	0.009
Free VEGF	0.047	0.204	0.003	0.018	0.940	0.827	0.042	0.288	0.536	0.660	0.030	0.230	0.220	0.320	0.004	0.034	0.903	0.722	0.381	0.556	0.066	0.262
sVEGFR-1	0.040	0.050	0.750	0.702	0.001	0.033	0.354	0.538	0.028	0.039	0.328	0.663	0.847	0.071	0.224	0.275	0.050	0.168	0.349	0.744	0.028	0.035
VEGFR-2	0.316	0.883	0.680	0.713	0.210	0.506	0.317	0.650	0.773	0.918	0.628	0.227	0.162	0.078	0.204	0.796	0.987	0.907	0.237	0.142	0.826	0.315
S/V ratio (0.5)	0.008	0.0002	0.404	0.067	0.0001	0.0002	0.074	0.024	0.012	0.013	0.006	0.008	0.001	0.0001	0.004	0.007	0.012	0.012	0.057	0.530	0.0007	0.0001

The univariate analysis for DFS and OS was performed for subgroups determined by ER status (cutoff value 10 fmol mg^{-1} protein), PgR status (cutoff value 10 fmol mg^{-1} protein), node involvement (positive or negative), TP expression level (cutoff value 194.9 ng mg^{-1} protein) and Her-2/neu expression level (cutoff value 13.5 ng mg^{-1} protein). The cutoff values for total VEGF, free VEGF, sVEGFR-1, VEGFR-2 and S/V ratio were 0.532 ng mg^{-1} protein, 0.135 ng mg^{-1} protein, 0.435 ng mg^{-1} protein, 0.105 ng mg^{-1} protein and 0.5, respectively. The listed values are *P*-values calculated using the log-rank test. All *P*-values are two-sided.

sVEGFR-1 expressions and its expressions were more dominant than VEGFR-1 expressions, and the concentration of sVEGFR-1 measured by ELISA was well correlated (data not shown). With samples treated without detergent, VEGFR-1 band was not detected in all cases. Although we have not examined all the cases that we used in the current study by Western blot analysis, the measurement of sVEGFR-1 expression with appropriate lysis buffer is meaningful with this ELISA system.

Total VEGF was determined to be a potent and independent prognostic indicator in both node-negative and node-positive cancers as reported in the previous studies (Toi *et al*, 2001; Ferrara *et al*, 2003). No significant prognostic value of free VEGF was observed in this study. It is difficult to explain why only total VEGF provides significant prognostic value but not free VEGF. Total VEGF concentration is a useful marker for predicting survival or disease progression. The current study using a highly sensitive sVEGFR-1 ELISA system confirmed that sVEGFR-1 is a significant prognostic indicator. In particular, we discovered that low sVEGFR-1 was related to an unfavourable prognosis, which was a slightly different result from that we had in a previous study with a relatively low-sensitivity sVEGFR-1 ELISA system, where we found a prognostic value of high sVEGFR-1 concentrations for favourable prognosis (Toi *et al*, 2002). The significance of the ratio of sVEGFR-1 to total VEGF (S/V ratio) as a prognostic marker was reconfirmed in this larger size of analysis. Particularly, a low S/V ratio was associated with unfavourable prognosis. Since sVEGFR-1 protein is estimated to be produced by both tumour cells and stromal cells in breast cancer microenvironment, it would be important to analyse the regulatory mechanisms of this balance more thoroughly.

In the subgroup analyses, we found that low sVEGFR-1 expression was significantly related to poor prognosis for ER-negative subgroup but not for ER-positive subgroup. The prognostic value of S/V ratio also significantly associated with ER negativity, and the correlation was more relevant than sVEGFR-1 status alone. It is reported that total VEGF expression is related to a poor prognosis in ER-positive patients rather than ER-negative patients (Linderholm *et al*, 2000; Foekens *et al*, 2001; Buteau-Lozano *et al*, 2002; Manders *et al*, 2003). Therefore, it is important to consider why the prognostic value of sVEGFR-1 or S/V ratio was associated with ER-negative status. Several explanations might be possible. First, the sensitivity of endothelial cells to VEGF might be different between ER-positive tumours and ER-negative tumours. Several reports discussed that ER-positive and ER-negative tumours display remarkably different gene expression phenotypes (Gruvberger *et al*, 2001). Also, ER-negative tumour cells produced larger amounts of growth factors and cytokines that can stimulate various types of cells including endothelial cells (Bando *et al*, 2003). Second, adjuvant therapies, especially post-operative hormone therapy, might cause a difference in the survival analysis between ER-positive and ER-negative subgroups. It is known that hormone treatments such as tamoxifen can downregulate VEGF expression in hormone-sensitive breast tumour cells or tumour tissues (Garvin and Dabrosin, 2003). In addition, it was recently documented that sVEGFR-1 was inducible in normal breast cell line and human breast cancer cell line, MCF-7, with hormone-dependent property in response to anti-oestrogen treatments (Elkin *et al*, 2004). In those cells, oestrogen was a potent downregulator of sVEGFR-1, and ER antagonism blocked the action dramatically. In the present study using nontreated primary tumours, sVEGFR-1 levels were significantly higher in PgR-negative tumours rather than PgR-positive tumours, which seems to support the idea that sVEGFR-1 is downregulated in purely hormone-dependent tumours such as ER-positive and PgR-positive tumours. According to these data, it might be possible to hypothesise that adjuvant hormonal treatments may suppress disease progression in patients having low S/V ratio, which is basically associated with poor survival, by modulating those

Table 5 Multivariate analysis of clinico-pathological and tumour biologic factors for DFS and OS

	DFS			OS		
Parameter	**Hazard ratio**	**95% CI**	***P***	**Hazard ratio**	**95% CI**	***P***
Nuclear grade I	0.172	0.041–0.598	0.015	0.132	0.016–0.861	0.034
Tumour size <5 cm	0.391	0.215–0.713	0.002	0.502	0.261–0.965	0.038
Node negative	0.269	0.121–0.598	0.001	0.3	0.136–0.656	0.002
VEGF-A (<0.532 ng mg^{-1} protein)	0.458	0.257–0.814	<0.001	0.499	0.256–0.972	0.043
sVEGFR-1/total VEGF-A >0.5	0.225	0.107–0.472	0.002	0.295	0.131–0.666	0.003

Hazard ratio indicates Cox model proportional hazard ratio; *P* is the Wald model *P*-value.

expressions. In ER-negative patients, theoretically it does not happen. Many other possibilities could be raised to explain this translational research question.

Another important finding observed in subgroup analyses is that the total VEGF and sVEGFR-1 levels as well as the S/V ratio exhibited significant prognostic importance for low Her-2/neu tumours but not for high Her-2/neu tumours (Table 4). Transfection of the Her-2/neu gene enhances VEGF expression in breast cancer experimental models (Yen *et al*, 2000). In this study, the intratumoral concentrations of Her-2/neu were significantly correlated with those of VEGF. The regulatory mechanism of VEGF and sVEGFR-1 expressions could be different between Her-2/neu-positive and Her-2/neu-negative subgroups.

As to the regulatory mechanism of sVEGFR-1, several factors such as growth mediators and hypoxia are reported to induce the expression of VEGFR-1 and sVEGFR-1 in endothelial cells (Barleon *et al*, 1997). Among these inducers, hypoxia might be a key factor especially, because it is capable of regulating the expressions of multiple angiogenesis-related molecules simultaneously (Griffiths *et al*, 1997; Bando *et al*, 2003). It was found in this study that the intratumoral concentration of sVEGFR-1 significantly correlated with those of VEGFR-2, total VEGF, free VEGF and TP. Therefore, it is interesting to know the relationship with the markers of hypoxia in future analysis, and to understand the machinery of alternative splicing of sVEGFR-1 in both tumour-associated stromal cells and hormone-dependent cancer cells.

In conclusion, the intratumoral concentration of sVEGFR-1 and VEGF and the ratio of sVEGFR-1 to total VEGF were potent prognostic indicators in 202 primary breast tumours in our study. The expression level and the balance between VEGF and sVEGFR-1 molecules were thought to be important to understand the hormone dependency of breast cancer and the sensitivity or resistance to hormonal therapy. Recently, it was clinically demonstrated that anti-VEGF therapy brings survival benefit to cancer patients in colorectal cancer patients (Hurwitz *et al*, 2004). We speculate that the determination of VEGF and sVEGFR-1 will also be useful to distinguish anti-VEGF therapy-sensitive tumours from less sensitive tumours. Eventually, the quantification of VEGF and its related molecules will be important for understanding the tumour growth machinery, the disease progression and the survival prediction of primary breast cancer and for considering treatment strategies with hormonal therapies and antiangiogenesis therapies.

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Thymidine phosphorylase (platelet-derived endothelial-cell growth factor) in cancer biology and treatment

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Thymidine phosphorylase (TP) is often induced in the tumour microenvironment by physiological and chemical stress. Its induction protects cells from apoptosis and helps cell survival by stimulating nucleoside metabolism and angiogenesis. Chemotherapy often upregulates TP, which acts in cell rescue; this result indicates that TP is a crucial therapeutic target. Clinical trials for metastatic diseases have shown that TP-targeting chemotherapy with fluorouracil derivatives greatly improves the effectiveness of conventional chemotherapy for not only response but also prognosis. This new idea, the improvement of TP-inducible therapy with TP-targeting therapy, should be further investigated for early disease states, and inhibitors of TP warrant extensive investigation.

Thymidine phosphorylase (TP) is a nucleoside metabolism enzyme associated with the maintenance of healthy mitochondria and the recovery of cells from pathological stress. Although TP has been studied for several decades, its research continues to provide many new and important aspects in cancer research and treatment.

In the 1970s, TP was purified from both *Escherichia coli* and *Salmonella typhimurium*. Investigators have since reported that it is a homodimer of 45 kDa subunits.[1] In the 1980s, a molecule extracted from human platelets was seen to have angiogenic effects and became known as platelet-derived endothelial-cell growth factor (PD-ECGF).[2] PD-ECGF showed significant angiogenic activity in vivo but no mitogenic activity in vitro. PD-ECGF was later recorded to have similar effects to those of TP. Additionally, the angiogenic activity of 2-deoxy-D-ribose—a thymidine metabolite—was also reported, in which it stimulated chemotaxis of the endothelium.[3] Transfection of PD-ECGF was also shown to significant increase TP activity and alter transfected cells angiogenically.[4] Subsequently, PD-ECGF and TP were confirmed as the same molecule.

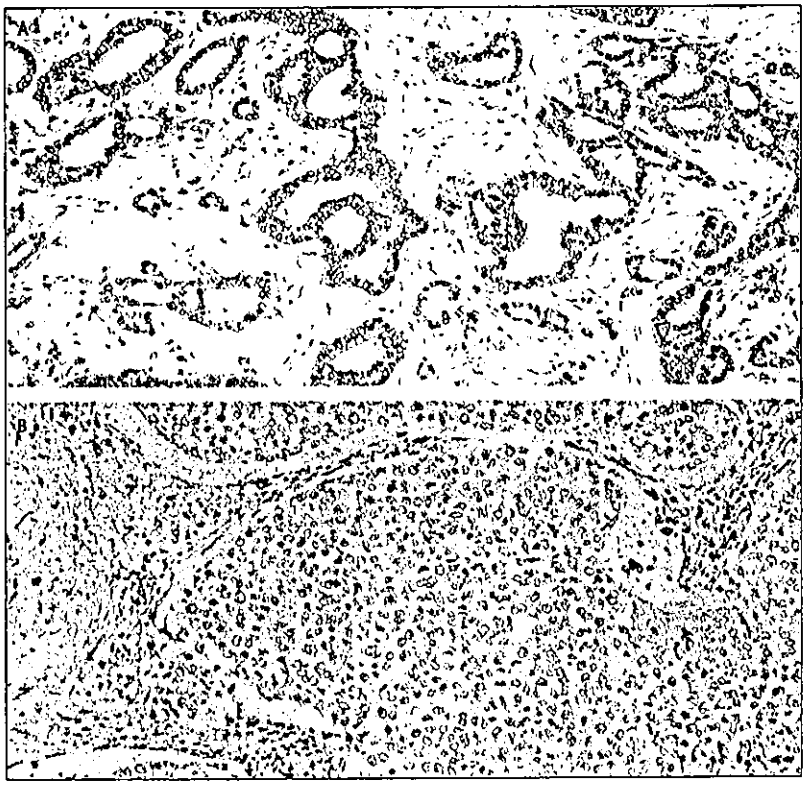

Figure 1: Immunostaining of TP in primary breast-cancer tissues
TP expression (brown) is dominant in (A) tumour cells and (B) in tumour-stromal cells.

Selective overexpression of TP in human cancer tissues has been investigated from various aspects. Initially, research on nucleoside metabolism enzymes showed that TP expression is frequently raised in a wide range of human cancer tissues (figure 1).[5] Expression is tumour dependent, and depends on the presence of other nucleoside metabolism enzymes such as thymidylate synthase. Somatic mitochondrial-DNA point mutations with TP deficiencies have also been reported,[6] resulting in mitochondrial neurogastrointestinal encephalomyopathy, with symptoms of gastrointestinal dysmotility, peripheral neuropathy, myopathy, and leucoencephalopathy. Therefore, TP is regarded as important in the maintenance of a balanced deoxyribonucleoside triphosphate (dNTP) pool for DNA replication and repair. Furthermore, investigations have clarified that TP acts as an antiapoptosis factor.[7–9] Because TP expression is very low in healthy human tissues, overexpression of the enzyme in cancer tissues has been associated with the promotion of tumorigenesis, whereas its genetic deletion or pharmacological inhibition might suppress tumour progression.

TP was shown to be associated with the anti-apoptosis mechanism, especially in cells damaged by stress, caused by factors such as hypoxia[10,11] or chemotherapeutic drugs.[12] Promotion of angiogenesis and inhibition of apoptosis by TP might explain why tumours that overexpress the enzyme have unfavourable prognosis. TP expression was induced by anticancer treatments in malignant cells and tissues,[13–16] which showed that the enzyme functions in the recovery of cells from damage caused by therapy. Various types of anticancer treatments such as

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48 Petit JY, Garusi C, Greuse M, et al. One hundred and eleven cases of breast conservation treatment with simultaneous reconstruction at the European Institute of Oncology (Milan). *Tumori* 2002; 88: 41–47.

49 Newman LA, Kuerer HM, McNeese MD, et al. Reduction mammoplasty improves breast conservation therapy in patients with macromastia. *Am J Surg* 2001; 181: 215–20.

chemotherapy[13–15] and radiotherapy[16] are known to stimulate TP expression. Therefore, suppression of TP has been postulated to slow down or suppress the recovery of cancer cells from this damage. Results from clinical trials suggest that the combined effects of TP-inducible and TP-targeting chemotherapies such as fluorouracil derivatives were synergistic in the antitumour response and in survival, supporting this recovery hypothesis. New inhibitors that can directly block activity of TP in cancers need to be developed.

Mechanisms of TP upregulation in cancer

Cancer cells contain large amounts of inflammatory mediators and are associated with physical stress. Even in the early stages of disease, such as for non-invasive or mucosal cancers, cytokine-rich conditions and hypoxic microenvironments are constructed. The up-regulation of such mediators and the involvement of stress cause additional changes in the genomic or proteomic regulation in tumour cells, and affect the phenotype of neighbouring stromal cells. Several cytokines such as tumour necrosis factor (TNF) α, interleukin 1, and interferon δ can upregulate TP expression in malignant cells.[17–19] Induction of TP by TNF α was mimicked by an antibody to the TNF-α receptor R2, but not by an antibody to TNF-α receptor R1, which suggested that TNF-α receptor R2 has a role in the regulation of TP expression.[20] Hypoxia or hypoglycaemic conditions could stimulate the expression of TP,[21] which indicates that the enzyme is a product of inflammation or microenvironmental stress (figure 2).

According to research on the interactions between TP and molecules related to inflammation, angiogenesis, and hypoxia in malignant tissues or chronic inflammatory disorders (such as inflammatory bowel diseases), TP expression was positively associated with TNF α and interleukin 1,[17] vascular endothelial growth factor (VEGF),[22–24] and hypoxia-inducible factor (HIF) 2α,[25] but was inversely related to angiopoietin (ANG) 1.[26] In particular, major inflammatory cytokines are strongly associated with TP expression.

Transcriptional control of TP during pathological conditions is still unclear. The human epidermoid carcinoma cell line A431 was reported to express 3·0 kb and 3·2 kb transcripts of TP containing long 5′ leader sequences of 1·5 kb and 1·7 kb, respectively, in addition to the originally identified 1·8 kb TP transcript.[27] The long 5′ leader sequence was seen to contain seven of eight copies of binding sites of the transcription factor SP1 that are present in the transcription promoter region of the 1·8 kb transcript. Amplified TP activity accompanies raised mRNA concentrations of the enzyme, but with no increase in mRNA stability, and the SP1 family has an important role in the induction of TP expression.[28] In deletion and mutation experiments of transcription assays of colon-cancer cell lines, expression of TP induced by TNF α fell when point mutations were made in three of four SP1 sites.[28]

Antitumour treatments are also inducers of TP. Chemotherapeutic drugs,[13–15] such as taxanes,[13,14] cyclophosphamide,[13,14] and oxaliplatin,[15] or irradiation[16] can induce expression of the enzyme in various types of tumours. An indirect pathway thorough the induction of inflammatory cytokines has been considered in the upregulation of TP; however, a direct mechanism could also be possible because several chemotherapeutic drugs are known to activate transcriptional factors (including SP1) directly.

Angiogenic activity

In nude mice, tumour cells that overexpress TP grow faster and form more angiogenic tumours than do

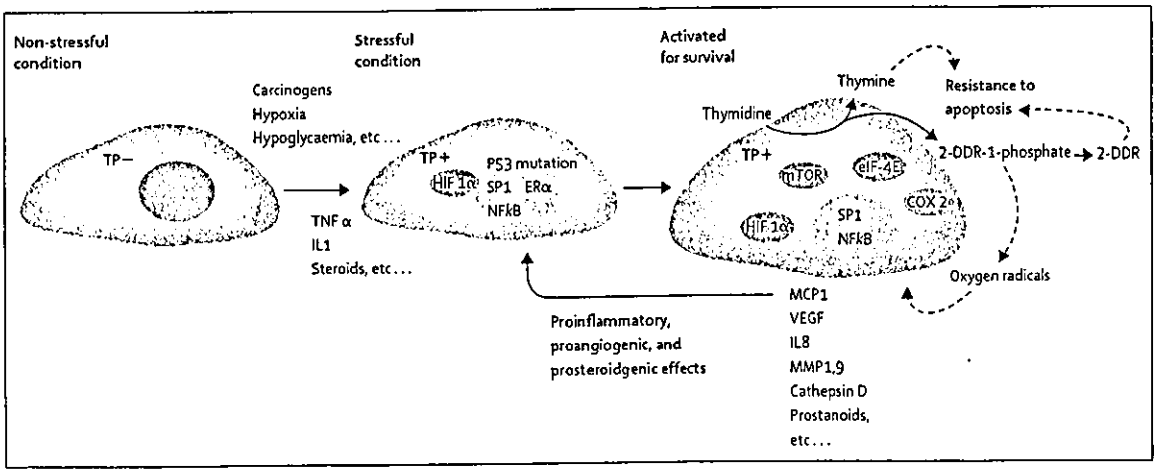

Figure 2: Proinflammatory and proangiogenic interactions of TP in tumour microenvironment
TP+=tumour cells overexpressing TP. TP−=wild-type tumour cells. HIF=hypoxia-inducible factor. NF=nuclear factor. mTOR=mammalian target of rapamycin (sirolimus). eIF=eukaryotic initiation factor. COX2=cyclo-oxygenase-2. MCP=macrophage chemoattractant protein. VEGF=vascular endothelial growth factor. MMP=matrix metalloprotease. ERα=oestrogen receptor α. 2-DDR=2-deoxy-D-ribose.

wild-type (TP–) tumour cells.[29] Overexpression of the enzyme correlates with intratumoural microvessel density[22–24] and inversely with the apoptosis index in human tumour tissues.[7–9] The angiogenic activity of TP is a major reason why TP-expressing tumours are less apoptotic and offer a poor prognosis. Additionally, the enzyme is frequently coexpressed with other proangiogenic molecules such as VEGF, which is an important mediator of vascular development, growth, permeability, and survival in human cancer tissues. The thymidine metabolite 2-deoxy-D-ribose is unique because it does not stimulate mitogenic activity but stimulates cell-migration activity in the endothelium.[3] No other thymidine metabolites apart from 2-deoxy-D-ribose seem to be angiogenic.

Hotchkiss and colleagues[30] stated that TP and 2-deoxy-D-ribose stimulated the formation of focal adhesions and Tyr397 phosphorylation of focal adhesion kinase and cell-surface integrin $\alpha_5\beta_1$ in endothelial cells in the human umbilical vein. Endothelial cell migration induced by TP was inhibited by antibodies directed against either $\alpha_5\beta_1$ or $\alpha_v\beta_3$, whereas VEGF-induced endothelial cell migration was blocked only by the $\alpha_v\beta_3$ antibody. This finding suggested that TP and 2-deoxy-D-ribose have different roles from VEGF in such migration. Presumably, 2-deoxy-D-ribose cooperates with VEGF in endothelial stimulation, because most tumours that coexpress TP and VEGF are highly angiogenic. Additionally, aortic ring formation and endothelial cell migration induced by 2-deoxy-D-ribose were blocked completely by sirolimus, which suggested that p70/S6 kinase activation is implicated in such endothelial stimulation.[31] The effectiveness of the so-called targets of rapamycin (sirolimus) inhibitors in angiogenesis induced by 2-deoxy-D-ribose needs to be established.

TP and VEGF are coexpressed in various human cancers.[22–24] These two potent angiogenic molecules, which have different specificities with respect to endothelial stimulation, could have a cooperative role in neovascularisation, according to research focusing on hypoxia. In analyses with clinical biomaterials of endometrial cancers, the transcriptional activity of HIF 1α is associated more with VEGF, whereas HIF 2α is associated more with TP.[25] Nevertheless, the mechanisms of TP induction through HIF 2α activation are still unknown. In analyses with vascular modelling or remodelling regulators such as ANG1, ANG2, and angiopoietin 4, TP mRNA expression was inversely related to ANG1.[26] Since ANG1 is also responsive to hypoxia, the transcriptional control of TP by hypoxia is different from that of angiopoietin 1. However, since several reports[32–34] have shown that even TP-induced tumours do not lead to any increase in the pace of growth in nude mice,[32–34] we need definitive data to reach any conclusion about the mechanisms taking place in neovascularisation.

TP as a marker of protumour stroma

TP expression in tumour-stromal cells such as tumour-associated macrophages seems to be a marker indicating the property of the tumour stroma. Clinical findings have shown that TP expression in these cells correlates greatly with unfavourable prognosis in various cancers.[35–37] Studies of multiple soluble mediators in tumour microenvironments have shown that TP often coexpresses with other inflammation-related or angiogenesis-related molecules. In clinical studies of breast cancers, TP was coexpressed with VEGF,[22–24] TNF α, interleukin (IL) 1,[17] and macrophage chemoattractant protein (MCP) 1,[38] whereas TP expression was inversely associated with that of IL12, a T-helper-1-type cytokine.[39] Expression also correlated greatly with aromatase expression in breast cancer tissues, suggesting that TP also accelerates tumour growth through a link with the oestrogen synthesis system.[40] Further, coexpression of TP and MCP1 has been seen in oesophageal cancer.[41]

TP is expressed not only in tumour cells but also in stromal cells. Several investigations have suggested that such expression in stromal cells is associated substantially with an unfavourable prognosis in various tumours such as breast cancer,[35] astrocytic tumours,[36] and uterine endometrial cancers.[37] Phenotypic expression of TP in tumour-stromal cells such as tumour-associated macrophages might be useful to assess their properties with respect to antitumour or protumour characteristics. As to the mechanisms of TP involvement in protumour–stroma formation, oxygen radicals seem to have a crucial function (figure 2). Metabolites of thymidine, including 2-deoxy-D-ribose-1-phosphate, and oxygen radicals seem to result in the production of various angiogenic, inflammatory, tissue-digestive, and steroidogenic molecules, which recruit responsive stromal cells and change their property from non-protumour or antitumour to protumour.[42]

Studies of these chain reactions and the education of stromal cells by tumour cells in local microenvironments are crucial to our understanding of how tumours acquire efficient and continuous growth systems. On the basis of these findings and hypotheses, we could regard the conversion of TP-positive to TP-negative stroma as treatment for cancer, in which protumour stroma might change back to antitumour stroma. The possibility that TP is a marker of tumour stroma still needs to be analysed thoroughly by the use of DNA microarray assays or proteomics for universal gene or protein expression to identify the role of all factors involved, some of which have only been suggested at present.

Antiapoptotic activity

TP provides an antiapoptotic function to cancer cells (figure 3). Kitazono and colleagues[10] showed that

transfection of TP into KB cells renders them resistant to apoptosis caused by hypoxia. 2-deoxy-D-ribose prevented hypoxia-induced apoptosis and 2-deoxy-L-ribose abrogated the effects. This mechanism implicates caspase-3 activation, mitochondrial cytochrome-c release, BCL2 and BCLXL downregulation, HIF-1α upregulation, and loss of mitochondrial transmembrane potential.[11] TP also causes resistance to apoptosis induced by chemotherapy such as cisplatin.[12] Notably, the researchers[12] reported that mutant TP with no enzymatic activity also suppressed cisplatin-induced apoptosis, which indicated that the enzyme is cytoprotective not only through its metabolites but also by a direct mechanism.

TP also seems to be important in the process by which tumour cells escape from immune surveillance by inhibiting Fas-induced apoptosis, and this role might be independent of TP enzymatic activity.[43] Haeme oxygenase 1 (HO1), which is closely associated with TP in gene regulation, might also be implicated in the antiapoptotic activity of cells that overexpress TP.[44] Although the details of these mechanisms are still unclear, TP seems to be crucial in the escape of tumour cells from various apoptotic signals such as physical stress, immune surveillance, and anticancer treatments.

TP induction by anticancer treatments

Various chemotherapeutic drugs can stimulate TP expression in many types of animals with tumours.[13–15] Irradiation is also known to stimulate induction of the enzyme.[16] According to a time-course study of tumour tissues, several inflammatory cytokines such as TNF α are upregulated first before TP is upregulated,[17] which suggests the possibility of an indirect TP induction mechanism. As a result, upregulation of the enzyme might be regarded as a type of protumour reaction. Therefore, understanding details of this pathway is crucial, especially in human cancer tissues, and new methods should be considered to suppress or control the reaction.

We retrospectively assessed changes in TP expression by anticancer treatments in primary breast tumour tissues in a neoadjuvant setting. Our results showed that therapies of doxorubicin with cyclophosphamide, epirubicin with cyclophosphamide, and docetaxel stimulated TP induction greatly in both tumour and stromal cells, even 3 weeks after the last doses were given.[45] By contrast, combination treatments of neither fluorouracil, doxorubicin, and cyclophosphamide, nor fluorouracil, epirubicin, and cyclophosphamide did not affect TP expression, which suggests that changes in the dNTP pool might be important in TP upregulation.

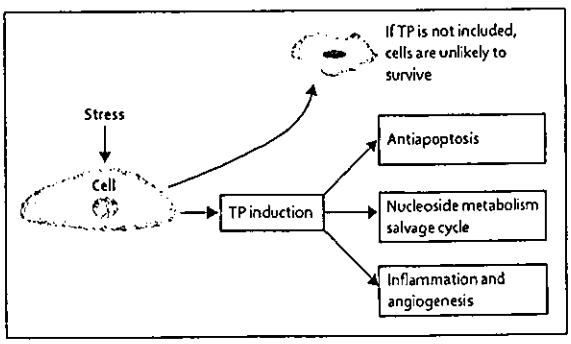

Figure 3: TP enzymic activity for survival from stress
TP stimulates nucleoside metabolisms, promotes angiogenesis, and protects apoptosis.

TP method	Treatment	Results	Ref
Breast			
Metastatic			
ICA	Containing doxofluridine	TP-high tumours more sensitive than TP-low tumours	48
Advanced			
HPLC	Doxofluridine	TP-high tumours more sensitive than TP-low tumours	49
Adjuvant			
ELISA	Cyclophosphamide, methotrexate, and fluorouracil	Intratumoural TP content clinically significant marker of favourable prognosis	50
ICA	Doxofluridine	Tumour TP expression clinically significant marker of favourable prognosis	51
ICA	Cyclophosphamide, methotrexate, and fluorouracil	Tumour TP expression clinically significant marker of favourable prognosis	52
PCR	Cyclophosphamide, methotrexate, and fluorouracil	No correlation between TP and clinical outcome	53
Colorectal			
Advanced			
PCR	Fluorouracil, leucovorin	Tumour TP marker of unfavourable prognosis	54
Adjuvant			
ELISA	Doxofluridine	Tumour TP marker of favourable prognosis	55
Gastric			
Advanced			
ICA	Doxofluridine	TP-high tumours more sensitive to treatment than TP-low tumours	56
ICA	Containing fluorouracil	TP-high tumours more sensitive to treatment than TP-low tumours	57
ELISA	5′-deoxy-5-fluorouridine	TP-high tumours more sensitive to treatment than TP-low tumours	58
ICA	Capecitabine	TP-high tumours more sensitive to treatment than TP-low tumours	59
ICA	Fluorouracil, containing fluorouracil	No correlation between TP and clinical outcome	60
Adjuvant			
ICA	Tegafur, uracil-tegafur	Ratio of tumour-TP to dihydropysimidine dehydrogenase marker of favourable prognosis	61
ICA	Doxofluridine, fluorouracil	TP-high tumours were more sensitive to doxofluridine than fluorouracil	62
ICA	Fluorouracil, uracil-tegafur, and doxofluridine	TP-high tumours more sensitive to treatment than TP-low tumours	63
ELISA	Doxofluridine	Ratio of tumour-TP to dihydropysimidine dehydrogenase marker of favourable prognosis	64
Oesophageal (advanced)			
ICA	Containing fluorouracil	TP expression negatively associated with overall response rate	65
Renal (secondary)			
ELISA	In-vitro fluorouracil	No correlation between TP and fluorouracil sensitivity	66

HPLC=high-pressure liquid chromatography. IICA=immunohistochemical assay. ELISA=enzyme-linked immunosorbent assay.

Table 1: Predictive value of TP for TP-targeting anticancer treatment

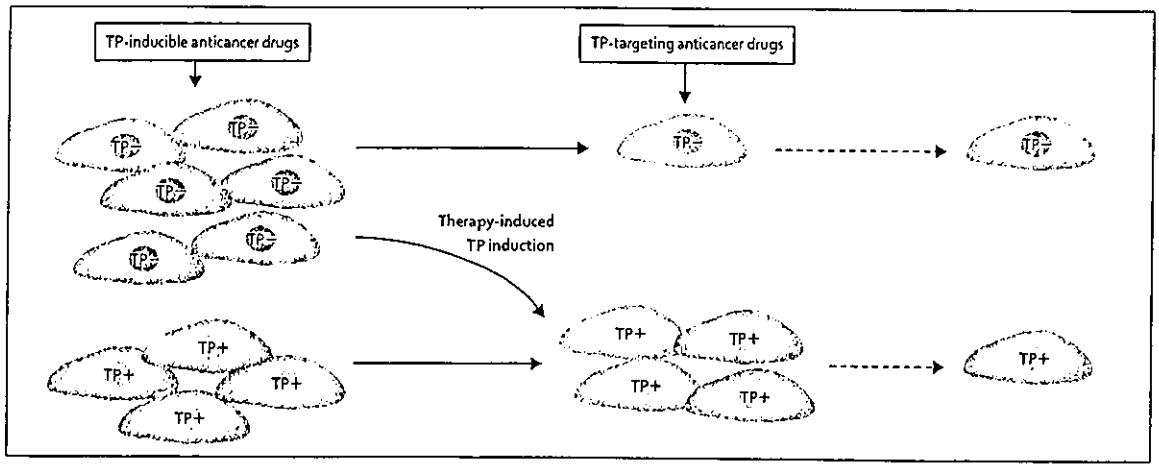

Figure 4: Model of TP-inducible chemotherapy enhanced with additional TP-targeting treatment
TP+=tumour cells overexpressing TP. TP−=wild-type tumour cells.

TP-targeting treatment

TP-related cancer treatment is unique because there are already TP-targeting treatments such as fluorouracil and its derivatives. In experiments of TP-transfected and TP-induced tumour xenografts in animals, fluorouracil,[46] doxifluridine,[13,14] and capecitabine[13,14] inhibited tumour growth strongly compared with controls, which indicated the TP-specific activity of these drugs.[47] Treatment with doxifluridine or capecitabine is especially specific because conversion of these drugs to their active form depends on TP. Predictive factor research on TP expression in cancer tissues for the clinical effectiveness of treatments of fluorouracil or its derivatives showed that these treatments were much more effective for TP-positive tumours than for TP-negative tumours (table 1).[48–66] Because overall antitumour response rates of capecitabine were 36% for breast cancer, 26% for colorectal cancer, and 34% for gastric cancer,[67–69] response rates of the TP-positive subgroup could be higher than these values. However, for all these studies mentioned, different non-standardised methods were used to measure TP concentrations; therefore, prospective studies with regulated methods should be used in the future.

Treatment	Response	Time to progression (median, months)	Overall survival (median, months)	Ref
Breast				
Previous anthracycline				
Capecitabine alone	36%	3·0	7·6	67
Capecitabine, docetaxel	42%	6·1	14·5	70
Capecitabine, paclitaxel	52%	8·1	16·5	71
Capecitabine, vinorelbine	40%	..	..	72
First-line				
Capecitabine alone	30%	4·1	19·6	73
Capecitabine, paclitaxel	51%	10·6	29·9	74
Capecitabine, vinorelbine	68%	9·3	..	75
Colorectal				
First-line				
Capecitabine, oxaliplatin	55%	7·7	19·5	15
Capecitabine alone	26%	4·6	12·9	68
Capecitabine, irinotecan	57%	7·8	16·8	76
Gastric				
First-line				
Capecitabine alone	34%	3·2	9·5	69
Capecitabine, cisplatin	55%	6·3	10·1	77
Capecitabine, docetaxel	60%	5·2	10·5	78

Table 2: Combination effects of conventional chemotherapy with TP-targeting treatment

Combination treatments of TP-inducible chemotherapy with TP-targeting chemotherapy

Since chemotherapeutic drugs, apart from fluorouracil, upregulate TP expression in cancer tissues, it is reasonable to postulate that the combination of TP-inducible chemotherapy with TP-targeting treatments could enhance the overall effectiveness of therapy (figure 4). Several experiments with animals have indicated that the combination results in a synergistic antitumour effect in human tumour xenograft models.[13–15]

Findings from clinical trials have also suggested that combination treatments between TP-inducible chemotherapy and capecitabine provided favourable effects for response rates, time to progression, and survival for patients with metastatic cancer compared with capecitabine alone (table 2).[15,67–78] For example, a large, randomised phase-III trial on metastatic breast cancer[70] showed improved survival and time-to-progression outcomes after the addition of capecitabine to standard treatments. Other clinical trials with regimens containing TP-inducible chemotherapy such as paclitaxel,[71,74] oxaliplatin,[15] cisplatin,[77] irinotecan,[76] cyclophosphamide,[79] mitomycin,[80] and irradiation,[81] have assessed their effects in metastatic settings. Furthermore, various combination treatments based on these results are

being used not only for metastatic disease but also for primary disease,[82] although the best timeframe for such combination therapies has not been fully investigated. Trials for primary cancers, especially for neoadjuvant therapy, should establish whether TP-targeting treatments act selectively for tumours that overexpress TP, either primarily or secondarily by anticancer therapy.

With respect to the possibility for TP inhibition, no drug is clinically available at present, although several drugs have been tested preclinically or clinically. Several competitive and non-competitive types of drugs have been isolated, and some have shown activity against angiogenic and antiapoptotic activities.[83–88] Since TP is implicated in premalignant inflammatory diseases such as gastritis induced by *Helicobacter pylori*,[89] these TP inhibitors might be useful for various purposes, including the prevention of cancer if the toxicity level is acceptable (table 3).[84–88] Combination treatment with TP-inducible chemotherapies might restrict the recovery of tumour cells from chemotherapeutic stress. However, combination therapies, including not only chemotherapy but also other types of cancer treatments such as radiotherapy and hormonal therapy, seem to be worth investigating. According to Nakajima and colleagues,[90] 2-deoxy-L-ribose inhibited the anti-apoptotic effects of its stereoisomer, 2-deoxy-D-ribose, and suppressed the growth and metastasis of TP-overexpressing KB cells that were transplanted into nude mice. Oral treatment of 2-deoxy-L-ribose greatly reduced the number of metastatic nodules in the liver and suppressed angiogenesis and enhanced apoptosis in metastatic nodules of KB cells overexpressing TP, which confirmed the importance of TP inhibition and indicated a new direction to control TP-induced protumour functions.[89]

Drug	Mechanism	Status	Comments
Doxofluridine	Converted to fluorouracil by TP	Approved in part of Asia and Italy	Tumour-targeting treatment using the difference of distribution of TP. Intermediate metabolite of capecitabine
Capecitabine	Converted to fluorouracil by TP in third conversion step	Approved for metastatic breast and colorectal cancer globally	Tumour-targeting treatment using the difference of distribution of TP
5-chloro-6[1-(2-iminopyrrolidinyl)methyl] uracil hydrochloride	Mimics substrate transition state	Clinical development	Suppressed TP-induced angiogenesis. Reversed TP-induced resistance to apoptosis
7-deazaxanthine (7DX)	Purine derivative, multi-substrate analogue inhibitor	Laboratory	Substantially suppressed angiogenesis in vivo
5'-O-trityl-inosine (KIN59)	Purine riboside derivative, non-competitive inhibitor	Laboratory	Inhibition of TP-induced angiogenesis
TAS-102 (alfa, alfa, alfa-trifluorothymidine and TPI)	Mixed-type competitive inhibitor	Laboratory	..
6-amino-5-chlorouracil	Mimics the substrate transition state	Laboratory	..

Table 3: TP-targeting treatments

Summary and future perspectives

Of the many nucleoside metabolism enzymes, TP is unique because it can both induce angiogenesis and protect cells from apoptosis. Since TP-targeting treatment has been developed in parallel with analyses of biological functions and translational implications, these therapies might become a specific chemotherapy method. For instance, the rate constant of doxifluridine breakdown was recorded as substantially faster in TP-overexpressing tumours than in controls in laboratory animals.[91] Further, the use of in-vivo, fluorine-19, magnetic-resonance spectroscopy to measure the pharmacokinetics of capecitabine and its intermediate metabolites in tumours could provide a non-invasive surrogate method to measure TP amounts in tumours and to predict the tumour response to capecitabine in individual patients. The best combinations between TP-inducible treatments and TP-targeting therapies seem to have improved response and survival in patients with various types of cancer. These treatments should be tested not only in metastatic disease but also in primary disease. For future TP-targeting therapies, new TP inhibitors need to be developed. These drugs can be used differently from conventional treatments in various situations.

Some issues still need to be resolved for TP-targeting treatments. First, standardised methods for the determination of TP have not been established. Second, all clinical results for TP as a predictive factor that we have shown are retrospective. Therefore, we should undertake prospective studies with the standardised methods for TP in the future. Alternatively, combination treatments with TP-targeting cytotoxic drugs and antiangiogenesis or anti-inflammatory therapies could be investigated further, since TP and multiple proangiogenic and proinflammatory molecules have been shown to be coexpressed.[92] These treatments might be able to change the property of tumour stroma to antitumour stroma. Finally, combination therapies with TP-targeting cytotoxic drugs and molecular-targeting drugs could be applied to individualised treatment by consideration of the predictive factors of respective treatments.

Search strategy and selection criteria

Data for this review were identified by searches of MEDLINE, PubMed, and references from relevant articles with the search terms "thymidine phosphorylase", "cancer", and "capecitabine". Abstracts and reports from meetings were included only if they related directly to previously published work. Only papers published in English were included.

Conflict of interest

We declare no conflicts of interest.

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