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ANGIOPOIETIN-2 EXPRESSION IN BREAST CANCER CORRELATES WITH LYMPH NODE INVASION AND SHORT SURVIVAL

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Angiogenic factors produced by tumor cells are essential for tumor growth and metastasis. In our study, the expression of Angiopoietin-1 (*ANG1*) and Angiopoietin-2 (*ANG2*) mRNA in archival human breast cancer tumor samples and in 6 breast cancer cell lines was investigated. Total RNA from biopsies of 38 breast cancer patients was extracted and *ANG1* and *ANG2* mRNA expression was measured by means of quantitative real-time RT-PCR (Taqman®). Matching data with available clinicopathologic and biochemical data revealed a significant association between *ANG2* expression and axillary lymph node invasion. Univariate and multivariate survival analysis, by means of Kaplan-Meier method and Cox's proportional hazards model, showed significant and independent association between *ANG2* mRNA level and both disease-free ($p < 0.0001$) and overall survival ($p < 0.0003$). An important fact is that, notwithstanding the small number of cases examined, this association was confirmed also in the group of lymph node-negative patients (DFS, $p < 0.003$; OS, $p < 0.020$). Immunohistochemical analysis demonstrated that Ang2 is expressed by both tumor cells and endothelial elements. Expression in tumor cells was confirmed by studying a panel of human breast carcinoma cell lines in culture by RT-PCR. In ZR75.1 and T47D cells, expression of *ANG2* mRNA was increased up to 10-fold by treatment with estrogen within 24 hr. Although preliminary, these data suggest a possible role of *ANG2* as a prognostic factor for primary breast cancer.

Key words: *angiogenesis; angiopoietin; breast cancer; real-time PCR; gene expression*

The concept that solid-tumor growth depends on angiogenesis is well established.[1] Tumor angiogenesis is a complex dynamic process consisting of extracellular matrix remodeling, endothelial cell proliferation and capillary differentiation, coordinated by several classes of ligands acting through cognate tyrosine kinase receptors. The most important pathways include the vascular endothelial growth factor (*VEGF*) family[2] and the Ang/Tie2 receptor family.[3] Tie2 is a tyrosine kinase receptor whose expression is largely restricted to endothelial cells[4–9] and has 3 known ligands: Angiopoietin-1 (*ANG1*), Angiopoietin-2 (*ANG2*) and Angiopoietin-4 (*ANG4*).[10–13] Angiopoietin-1 and Angiopoietin-2 show comparable binding affinity for Tie2. However, while Ang1 induces receptor phosphorylation and activation, Ang2 does not and competitively inhibits Tie2 activation,[12] giving these ligands functionally opposing effects. Ang4 appears to act as an agonist similar to Ang1.[13] *VEGF* and the Angiopoietins play complementary and coordinated roles in the development of new blood vessels. Ang1 helps to maintain and stabilize mature vessels by promoting interaction between endothelial cells and supporting cells.[12,14,15] Conversely, Ang2 is only expressed at sites of vascular remodeling and is thought to counteract the stabilizing action of Ang1.[12] Destabilization by Ang2 in the presence of VEGF or other angiogenic factors has been hypothesized to induce an angiogenic response; in the absence of VEGF Ang-2 induces vessel regression.[11,12,16] It has been demonstrated that tumors initially coopt existing vessels to form a vascularized tumor mass; in this tumor mass there is a regression of these coopted vessels, leading to an avascular tumor, and the remaining tumor is rescued by angiogenesis at the tumor margin.[17] Neither Ang1 nor Ang2 has a proliferative effect on the endothelial cells, but Ang1 acts as a survival factor for endothelial cells.[18]

In breast cancer, several studies have suggested that the degree of vascularization of the primary tumor is a predictor of survival,[19,20] and tumor angiogenesis in invasive breast carcinoma is an independent and significant prognostic factor.[21–25] Recent studies have shown that the incidence of metastasis can be correlated with the number and density of blood vessels in breast, lung, prostate, esophageal, colon and gastric carcinoma and melanomas.[26–29] In some tumors presenting particularly high vascularization, as in the case of brain tumors, the expression of *ANG2* has been reported.[30–32] In addition, high *ANG2* expression was reported to correlate with shorter survival in gastric carcinoma.[33] *ANG2* mRNA expression was found also in breast cancer,[34,35] where it appears related to *ERBB2* oncogene overexpression.[35]

To establish whether angiopoietin expression may contribute to breast cancer progression, in our study we evaluated the expression of *ANG1* and *ANG2* mRNA by quantitative RT-PCR in human breast cancer tumor samples and examined their correlation with clinical data. In addition, the possible regulation of *ANG2* expression by estrogen was examined in the human breast carcinoma cell lines T47D and ZR75.1.

MATERIAL AND METHODS

Cell cultures and RNA extraction

Human breast cancer ZR75.1, SKBR3, T47D, MDA.MB.231 and MCF7 cells were grown in DMEM (Gibco, Paisley, Scotland)

Abbreviations: Ang, angiopoietin; Cat-D, cathepsin-D; ER, estrogen receptor; E2, 17-beta-estradiol; FAM, 6-carboxyfluorescein; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; PR, progesterone receptor; TAMRA, 6-carboxy-N,N,N′,N′-tetramethylrhodamine; *VEGF*, vascular endothelial growth factor.

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supplemented with 10% heat-inactivated FBS and antibiotics. The immortalized human breast epithelial cells MCF10A were grown in a DMEM-HAM F12 (1:1) medium, supplemented with 5% horse serum, insulin (10 μg/ml), epidermal growth factor (EGF) (20 ng/ml), cortisol (1.4 μM) and antibiotics, as previously described.[36] For estrogen treatment, ZR75.1 and T47D cells were grown in DMEM supplemented with 5% or 10% heat-inactivated FBS and antibiotics. Cells were plated at 20% confluence and maintained for 3 days in steroid-free medium (Phenol red-free DMEM medium, with 5% charcoal-stripped FBS and antibiotics), before stimulation with 50 nM (ZR75.1 for kinetic study) or 10 nM (T47D and ZR75.1) 17-beta-estradiol (E2). At the indicated times, cells were scraped on ice and stored at −80°C. Total cellular RNA was extracted using the guanidinium thiocyanate/acidic phenol extraction, as previously described.[37]

Clinical samples

Biopsies collected from breast surgery excision of 38 patients were kept in ice until pathologic analysis. Those resulted as breast ductal infiltrating carcinomas were frozen and stored into liquid nitrogen. Different sections of each tumor, as cut for pathologic examination, were pooled and pulverized with a Mikro-Dismembrator (B. Braun, Melsungen, Germany) in the presence of liquid nitrogen and then dissolved in a lysis solution (12 ml for 1 g of tissue) containing 25 mM sodium citrate, 0.5% Sarkosyl and 4 M guanidinium thiocyanate. Total RNA was extracted as above.

RT-PCR and real-time RT-PCR

Total RNA was pretreated with DNase I (Promega, Madison, WI) before retrotranscription. Reverse transcription reactions were done in 20 μl containing 1 μg of total RNA, 1 mM of each deoxynucleotide triphosphate (Promega), 200 units of MMLV reverse transcriptase enzyme (Promega), 2.5 μM of random primers (Promega), 5X RT-Buffer (Promega) and 40 units of ribonuclease inhibitor (Promega). To accomplish wide RT performance variations, 3 independent runs of RT were done starting from 1 μg total RNA and the cDNA mixed up. For the PCR reactions the following primers were used: *ANG1* 5′-AAAGGTCACACTGGGACAGC-3′ and 5′-TTCTGACATTGCGCTTTCAA-3′; *ANG2* 5′-TCCAAGCAAAATTCCATCATTG-3′ and 5′-GCCTCCTCCAGCTTCCATGT-3′; *VEGF-A* 5′-TACATCTTCAAGCCATCCTGTG-3′ and 5′-TTTGCAGGAACATTTACACGTC-3′; *VEGF-C* 5′-TGCCGATGCATGTCTAAACT-3′ and 5′-GTTGAGTCATCTCCAGCATCC-3′. The primers for *VEGF-A* amplify 4 isoforms of the gene. Amplicon sizes were 298 bp for *ANG1*, 300 bp for *ANG2*, 194 bp for *VEGF-C*, and the annealing temperature (Ta) was 56°C for *ANG1*, 60°C for *ANG2*, 55°C for *VEGF-A* and 54°C for *VEGF-C.* Amplification was carried out by 40 cycles, each lasting 30 sec at 95°C, 30 sec at Ta and 30 sec at 72°C.

Real-time quantitative RT-PCR analysis was performed using an ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA). Specific primers and probes for each gene were designed using the Primer Express 1.0 software (Applied Biosystems). Melting temperatures of primers and probe were 60°C and 70°C, respectively. Amplicon sizes were 73 bp for *ANG1* and 65 bp for *ANG2*. A 25 μl reaction mix contained 2× Universal Master Mix (Applied Biosystems), 100 nM TaqMan® probe, 20× PDAR (Pre-Developed TaqMan® Assay Reagents, Applied Biosystems) 18S, 300 nM both primers. After 2 min at 50°C to allow AmpErase uracil-N-glycosilase (UNG) to destroy potential contaminant PCR products and 10 min at 95°C to denature UNG and activate Taq polymerase, the amplification was carried out by 45 cycles, each of 15 sec at 95°C and 1 min at 60°C. All amplified samples were resolved in a 2% agarose gel to confirm the PCR specificity. The sequences of the PCR primer pairs and fluorogenic probe, respectively, that were used for each gene are as follows: *ANG1* 5′-CAGGAGGATGGTGGTTTGATG-3′, 5′-TGGTTTTGTCCCGCAGTATAGAA-3′ and 5′-FAM-TGTGGCCCCTCCAATCTAAATGGAATG-TAMRA-3′; *ANG2* 5′-TTCCTCCTGCCAGAGATGGA-3′, 5′-TGCACAGCATTGGACACGTA-3′ and 5′-FAM-ACTGCCGCTCTTCCTCCAGCCC-TAMRA-3′.

Threshold cycle, Ct, which correlates inversely with the target cDNA levels, was measured as the cycle number at which the reporter fluorescent emission increased above a threshold level. In order to obtain an absolute quantitation, a standard curve was created using plasmids containing cDNA of the target gene as reference samples, quantified by spectrophotometric analysis. For estrogen treatment experiments, relative quantitations were performed, *i.e.,* all data were referred to the level of target mRNA measured in untreated cells before treatment (point 0): first, each Ct value was corrected for the Ct of the 18S ribosomal RNA (Ctr), used as an internal reference and co-amplified with target, then Ct of each sample was subtracted from the Ct_0.

The relative quantity of the template (Q) was therefore calculated as: $Q = 2^{-(Ct-Ctr)-(Ct_0-Ct_{0r})}$. All samples were run in triplicate and the mean and standard deviation calculated: $Var = St.\ Dev.^2 = e^{-2\mu \ln 2+2\sigma^2(\ln 2)^2}\ [e^{\sigma^2(\ln 2)^2}-1]$ where μ is the mean of (Ct-Ctr)-(Ct0-Ct0r) and σ its standard deviation. For the expression of relative quantitation data, the lowest value is set equal to 1 and the others expressed as a ratio.

For real-time RT-PCR experiments on ZR75.1 cells, a panel of 11 possible reference genes was tested using the TaqMan® Human Endogenous Control Plate (Applied Biosystems), containing most common reference genes: acidic ribosomal protein, 18S rRNA, β-actin, cyclophilin, glyceraldehyde-3-phosphate dehydrogenase, beta-glucuronidase, TATA-binding protein, transferrin receptor, β-2-microglobulin, phosphoglycerokinase, hypoxanthine ribosyl transferase.

Immunohistochemistry

Immunohistochemistry was performed as previously described.[32] Deparaffinized sections were permeabilized in Triton 0.1% for 10 min at room temperature. They were saturated in blocking solution (DAKO A/S, Glostrup, Denmark) for 30 min and then incubated overnight at 4°C with goat polyclonal antibody against the C-terminus peptide of human Ang2 (1:200 in blocking solution, Santa Cruz Biotechnology, Santa Cruz, CA). Specificity of anti-Ang2 was tested[32] by incubating antibodies with the respective immunizing peptides before immunostaining. Biotinylated rabbit anti-goat IgG (1:400 in blocking solution, DAKO) was used as secondary antibody and proteins were detected using the streptavidin-peroxidase-complex (DAKO). To detect vessels and to exclude macrophages expression, a monoclonal anti-CD31 (DAKO) and a monoclonal anti-CD68 (DAKO) were used.

Statistical analysis

The analyses have been conducted using the SPSS v. 8.0 package program. Associations between the clinicopathologic data and gene expression were analyzed using the rank nonparametric statistical tests of Mann-Whitney and Kruskal-Wallis, since no evidence of normal distribution was available. The survival analysis was carried out in univariate and multivariate mode, using Kaplan-Meier method and Cox's proportional hazards model. Model selection was done using the forward stepwise method.

RESULTS

Expression of ANG1 *and* ANG2 *in tumor samples*

A preliminary screening on breast tumors RNAs by traditional RT-PCR showed that *ANG1* and *ANG2* mRNA can be easily detected in most cases and that both of them show a wide range of levels as compared to the internal reference *GAPDH* mRNA (Fig. 1). Semiquantitative evaluation by densitometry and normalization based on *GAPDH* band intensity allowed us to estimate that *ANG1* and *ANG2* expression in human breast cancer samples varies in a hundred-fold range. Such an elevated variability between samples is a prerequisite for a prognostic marker. Therefore, we set up to examine quantitatively the levels of *ANG1* and *ANG2* mRNA expression in representative cases of breast cancer. Thirty-eight

archival breast tumor tissues, diagnosed as ductal infiltrating carcinomas, with at least 7 years of follow-up, were randomly selected from our tissue bank. *ANG1* and *ANG2* were quantitatively measured by real-time RT-PCR, using co-amplified 18S rRNA as internal reference and absolute quantification using plasmids carrying full-length *ANG1* and *ANG2* cDNAs as reference standards. *ANG1* and *ANG2* quantitation were done on the same cDNA samples for each specimen. The expression range was $6.32 \times 10^3 - 314.7 \times 10^3$ target copies / μg RNA for *ANG1* (corresponding to 26.16–1303.2 equivalents to plasmid fg/μg RNA), and $0.44 \times 10^3 - 125.2 \times 10^3$ target copies / μg RNA for *ANG2* (corresponding to 2.40–678 equivalents to plasmid fg/μg RNA). The data obtained by traditional RT-PCR (Fig. 1) are in contrast with these values, since grossly equivalent levels are observed for both genes. Differences in the set of primers used and in the amplification conditions may explain this ambiguity. For *ANG2*, both the primer set used for conventional PCR and the one used for real-time were designed to detect all the isoforms available in public databases. In the case of *ANG1*, the primers used for real-time PCR were contained in the amplicon targeted for conventional PCR and both sets were able to detect all the isoforms, except the transcript variant 2 (gi: 21328450). Of course, the presence, in tumor tissues, of additional transcripts potentially affecting RT-PCR assays can not be excluded.

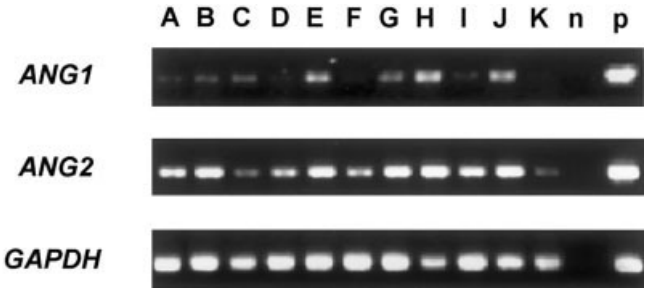


FIGURE 1 – RT-PCR analysis of *ANG1, ANG2,* as compared to *GAPDH*, in 11 representative samples of breast carcinoma (lanes A–K). Lanes n and p indicate the negative and positive controls, respectively.

Associations between ANG1 *and* ANG2 *expression and clinicopathologic and survival data*

ANG1 and *ANG2* values, considered as continuous variables, were compared to available clinicopathologic and biochemical data and subjected to statistical analysis to reveal significant associations. As shown in Table I, *ANG1* expression showed no significant correlation, while a significant positive association was found between *ANG2* levels and lymph node metastasis. Positive correlation between *ANG2* level and the number of vessels was observed, notwithstanding the small number of cases considered. Marginally significant correlation was also found with serum PSA (Kallikrein-3) ($p < 0.043$), which has been shown to carry independent information as a prognostic factor in breast cancer patients.[38–42] Association of *ANG1* and *ANG2* mRNA levels with survival time was examined by means of the Kaplan-Meier univariate survival analysis. An iterative best-fit method was used to select cutoff values for angiopoietin expression. *ANG2* levels higher than 2.66×10^3 copies/μg total RNA associated significantly with both shorter disease-free (DFS) and overall survival (OS), as shown in Figure 2*a* and *b*, respectively ($p < 0.0001$ for both). To assess whether *ANG2* carries a prognostic information independent from that provided by the established prognostic factors (nodal status, tumor size, grading and hormonal receptor status), a multivariate analysis by Cox's proportional hazards model was performed. Since either grading or estrogen receptor (ER) status were unavailable in 3 cases, multivariate analysis was

TABLE I – ASSOCIATION OF *ANG1* AND *ANG2* mRNA LEVELS WITH PATHOLOGIC AND CLINICAL DATA. THE NONPARAMETRIC MANN-WHITNEY AND KRUSKAL-WALLIS STATISTICS WERE USED AND THE MEAN VALUE REPRESENTS THE CENTRAL RANK OF EACH SERIES

Variables	Ang 1			Ang 2			Ang 2/Ang 1		
	N[1]	Mean rank value	*p*-value	N[1]	Mean rank value	*p*-value	N[1]	Mean rank value	*p*-value
pT			NS[2]			NS			NS
I	10	17.30		10	21.65		10	20.00	
II	22	18.77		22	16.32		22	17.59	
III–IV	5	23.40		6	27.58		5	23.20	
Lymph nodes			NS			0.005[3]			NS
Negative	23	17.35		23	15.43		23	16.43	
Positive	14	21.71		15	25.73		14	23.21	
Grading			NS			NS			NS
≤2	11	19.73		11	20.73		11	22.00	
>3	25	17.96		26	18.27		25	16.96	
ER			NS			NS			NS
<20%	15	17.67		15	20.43		15	20.13	
>20%	20	18.25		20	16.17		20	16.40	
PR			NS			NS			NS
≤20%	21	17.67		21	17.90		21	17.88	
>20%	13	17.23		13	16.85		13	16.88	
ERB-B2			NS			NS			NS
0%	5	9.60		5	8.40		5	8.80	
1–50%	11	10.36		11	9.55		11	9.45	
>50%	3	9.33		3	14.33		3	14.00	
Cat-D			NS			NS			NS
0%	2	4.00		2	2		2	7.50	
1–40%	9	10.67		9	10.33		9	8.33	
>40%	5	6.40		5	7.80		5	9.20	
Vessels[4]			NS			0.04[3]			NS
<85	2	6.00		2	2.00		2	4.50	
≥85	12	7.75		12	8.42		12	8.00	

[1]Where the total number is not 38, pathologic data are unavailable.–[2]NS, not statistically significant.–[3]Significant ($P < 0.05$).–[4]Vessels per mm^2.

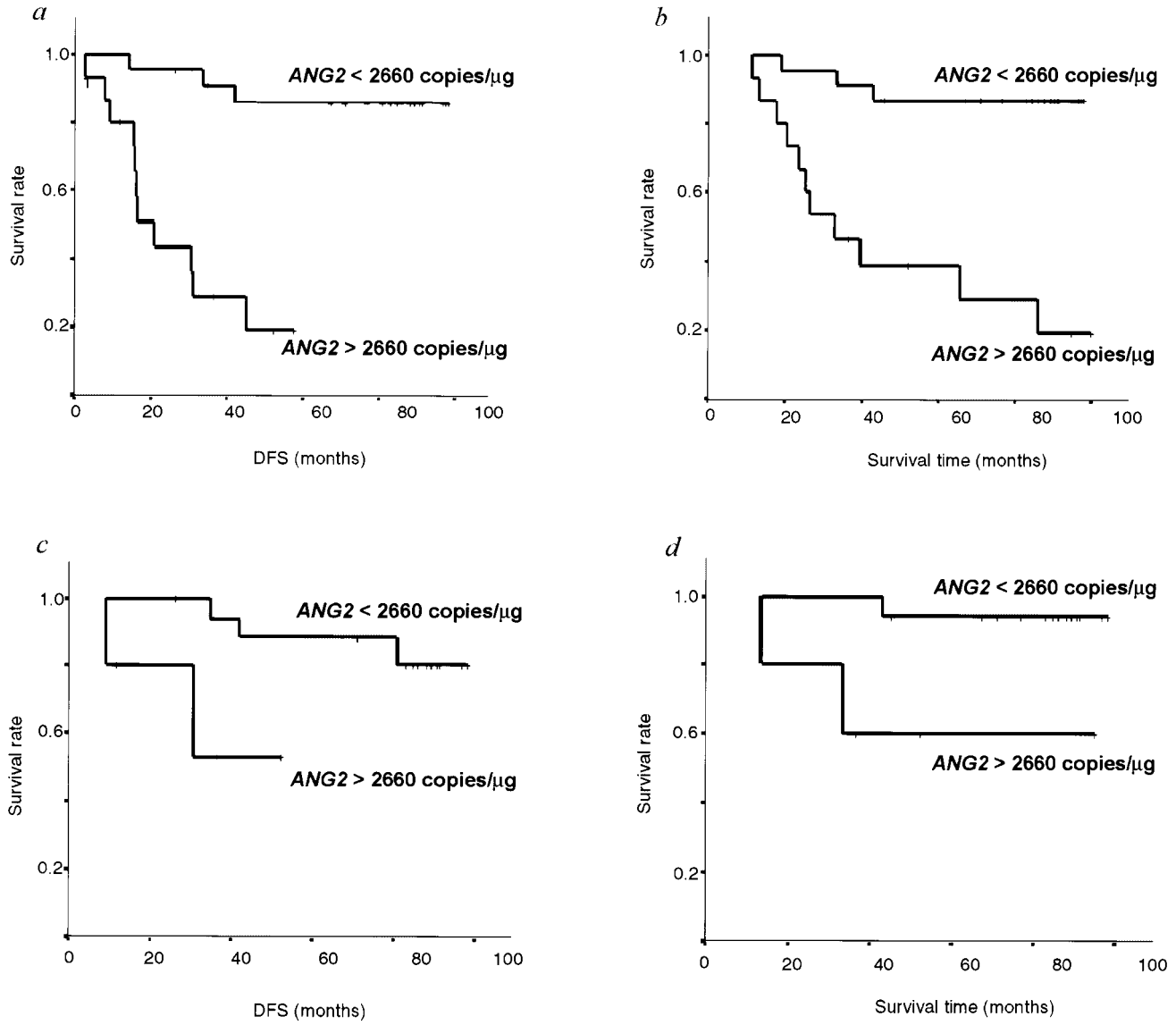


FIGURE 2 – Disease-free and overall survival Kaplan-Meier's curves of patients with breast carcinoma with regard to *ANG2* expression in tumor tissues. Disease-free survival *(a)* and overall survival *(b)* analyses show a significant correlation between *ANG2* expression and survival ($p < 0.0001$). *(c,d)* The Kaplan-Meier analysis in lymph node-negative patients; the association between high *ANG2* expression and survival holds true ($p < 0.0281$ for DFS; $p < 0.0189$ for OS).

performed on a total of 35 samples, among which 22 were lymph node-negative. *ANG2* conserved a significant association with survival in multivariate analysis ($p < 0.0001$ for DFS and $p < 0.0003$ for OS), giving a relative risk of 8.8 for DFS and 5.3 for OS. *ANG2* prognostic power was then analyzed in lymph node-negative (N-) and -positive (N+) subgroups and, as shown in Figure 2*c* and *d*, the association between high *ANG2* expression and survival held true also in the subgroup of patients with no axillary-node invasion ($p < 0.003$ for DFS; $p < 0.02$ for OS). *ANG1* mRNA levels did not show any association with survival in any of the tests performed (data not shown).

Immunohistochemistry

It is rather important to establish whether tumor cells express *ANG2* mRNA. In fact, since several articles reported *ANG2* expression in different cell types, it was of interest to learn which cells were responsible for the high expression levels seen in ductal carcinomas. This problem was approached by performing immunohistochemistry on tumor slices and by studying *ANG2* mRNA expression in cultured breast cell lines. Formalin-fixed, paraffin-embedded tissues from tumors previously characterized by real-time RT-PCR were stained with a specific Ang2 antiserum. Twelve samples were analyzed, 6 showing high levels of *ANG2* mRNA (range 29.1–5.83 $\times$ 10^3 copies/μg RNA) and 6 with low levels of *ANG2* mRNA (range 0.89–1.40 $\times$ 10^3 copies/μg RNA). To detect vessels and to exclude expression in macrophages, an anti-CD31 and an anti-CD68 were incubated on adjacent sections (data not shown). Ang2 immunoreactivity was present in all the high-level mRNA samples examined, whereas the low-level mRNA samples showed a weak positivity. An example of this is shown in Figure 3*a* and *b*. The tumor shown in Figure 3*a* contained 21.9 $\times$ 10^3 copies/μg of *ANG2* mRNA, as measured by real-time RT-PCR (it ranked fourth among all the tumors examined), while the specimen in Figure 3*b* had a level of 1.24 $\times$ 10^3 copies/μg (rank = 32nd). Immunohistochemistry staining revealed a pattern of Ang2 expression focally concentrated in some tumor areas

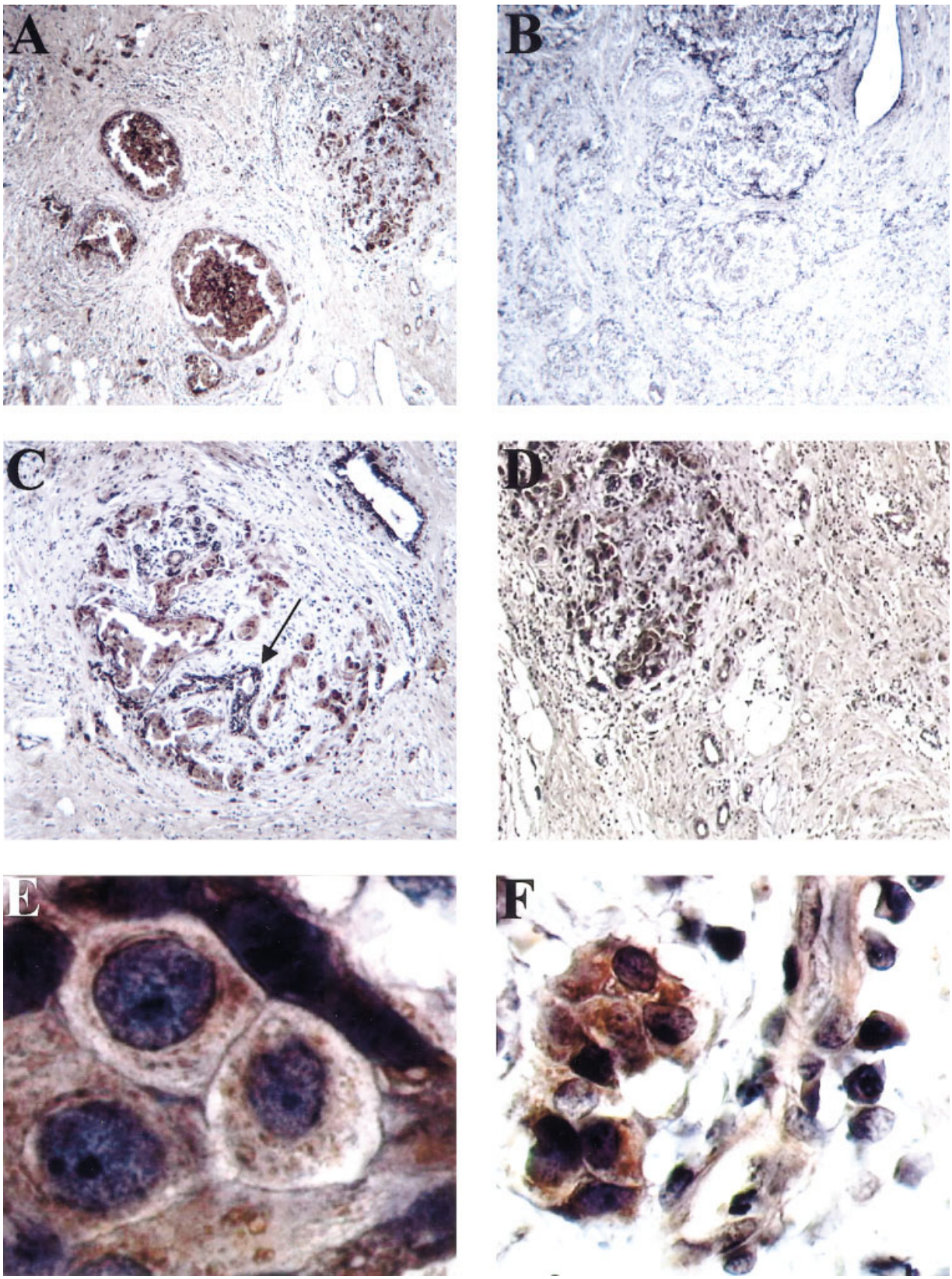


FIGURE 3 – Immunohistochemical analysis of Ang2 protein in representative breast carcinoma slices. High-level mRNA samples exhibit highly positive epithelial cells both in *in situ* and in invasive components *(a)* and positive endothelial cells *(d),* while low-level mRNA samples exhibit a weak staining *(b). (c)* The negative staining (arrow) of an area of normal tissue in the tumor. Original magnification 40× *(a–d). (e,f)* Higher magnification (100×) of positive tumor and endothelial cells.

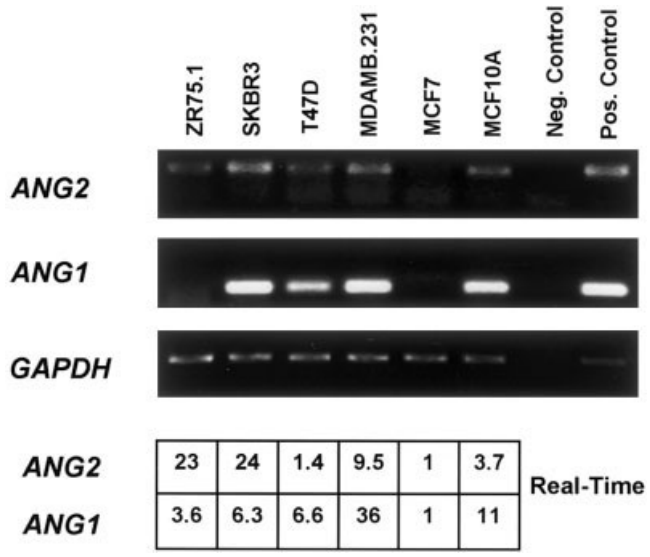
where epithelial cells are highly positive, as already shown in other cancers.[43] Quantitative RT-PCR data, though, are likely to represent average measures, since sections from different parts of the tumors were pooled for RNA extraction. Noteworthy, the *in situ* component of the tumors, when present, was stained at the same level as the invasive component, as exemplified in the tumor shown in Figure 3*a*. In some areas, in which both tumor cells and normal tissue were visible, we could not find convincing evidence of expression in the normal cells (Fig. 3*c* and data not shown). Ang2 expression was demonstrated in both tumor cells and endothelial cells (Fig. 3*d,f*). Ang2 immunoreactivity was seen in angiogenic and mature vessels, but it was consistently higher in vessels near the tumor, as in the case shown in Figure 3*d* and *f*. Figure 3*e* shows details of Ang2-positive tumor cells at higher magnification. Very interestingly, the protein is present in the cytoplasm of tumor cells within membrane vesicles, possibly indicating its secretory fate.

Expression of ANG2 *in human mammary cell lines*

Immunohistochemistry data clearly indicated that tumor cells, in addition to endothelial elements, express *ANG2*. To ascertain whether the ability to produce *ANG2* is a property intrinsic to transformed mammary cells, we analyzed 5 representative breast cancer cell lines and the nontumorigenic mammary cell line MCF10A, by means of both RT-PCR and real-time PCR. Results, shown in Figure 4, demonstrated that cultured mammary cells do express a variable amount of *ANG2*. A quite evident discrepancy exists between data from traditional end-point RT-PCR (upper) and real-time data (lower). This further confirms that traditional RT-PCR data should be taken only for their qualitative, but not quantitative, value.

Effect of estrogen on ANG1 *and* ANG2 *gene expression in ZR75.1 and T47D cells*

Because of the link between *ANG2* expression and clinical outcome and since all ER+ patients undergo antiestrogenic adjuvant therapy, it could be of interest to know whether *ANG2* expression is under estrogenic control. Indeed, among the examined cell lines, MCF-7, ZR75.1 and T47D are estrogen receptor-positive (ER+) and show variable amounts of *ANG2* mRNA. To investigate this issue, ER+ T47D and ZR75.1 cells were cultured in estrogen-deprived medium for 3 days to obtain ER replenishment, then treated or not with 10 nM 17-β-estradiol (E2) for 6, 12 or 24 hr. Real-time RT-PCR analysis was used to quantitate *ANG2* and *ANG1* mRNA levels. As shown in Figure 5*a*, in T47D cells there was a clear delayed induction of *ANG2* in estrogen-treated samples that is already appreciable after 12 hr but is markedly increased at 24 hr. In ZR75.1 cells (Fig. 5*b*), instead, a 6-fold induction by estrogen was already observed at 6 hr, and the induction declined at 24 hr. In both cell lines the *ANG2* expression level in nonestrogenic conditions increased with time. *ANG1*



ANG2	23	24	1.4	9.5	1	3.7	Real-Time
ANG1	3.6	6.3	6.6	36	1	11	

FIGURE 4 – RT-PCR analysis of *ANG1, ANG2* and *GAPDH* mRNA expression in 6 human mammary cell lines. End point RT-PCR analysis (upper) as in Figure 1 and quantitative real-time RT-PCR (lower) reanalysis of the same RNAs, setting the expression levels in MCF-7 cells as the reference value.

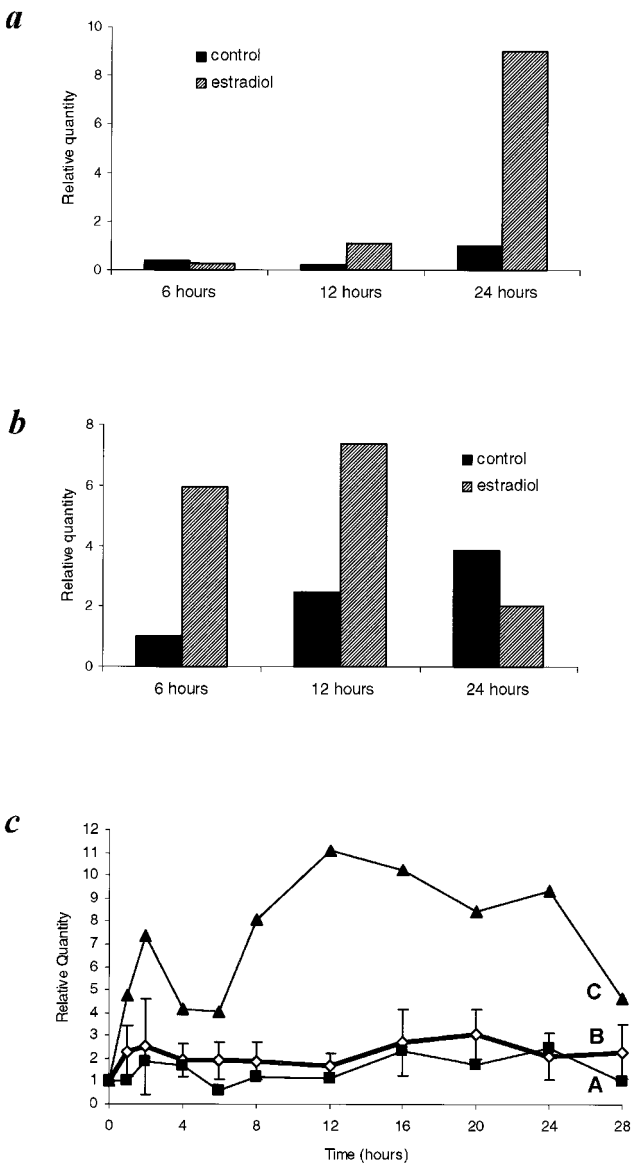


FIGURE 5 – Effects of 17-beta-estradiol treatment on *ANG2* mRNA level in 2 estrogen-dependent human breast cancer cell lines. T47D *(a)* and ZR75.1 *(b)* cells were treated with or without 10 nM E2 for the indicated times, and *ANG2* mRNA quantitated by real-time RT-PCR. *(c)* ZR75.1 cells were treated with 50 nM E2 for the indicated times. Real-time RT-PCR quantitations are shown for several genes and referred to the cells before treatment. The data are expressed as relative values referred to the 0 time point. Curve A (filled squares) and curve C (triangles) represent, respectively, *ANG1* and *ANG2* mRNA expression. Curve B (open squares) represents the average ratio between the different time points and time 0 of the expression of 11 housekeeping reference genes and their standard deviation. Standard deviation for *ANG1* and *ANG2* was within 10%.

mRNA levels were not changed by estrogen treatment (data not shown). Induction of *ANG2* mRNA in ZR75.1 cells was more rapid than in T47D. To further examine the kinetics of *ANG2* response to E2 in ZR75.1 cells, RNA samples derived from a different study, devised to explore the kinetics of genomic responses to E2 treatment in ZR75.1 cells on cDNA microarrays, were analyzed and *ANG2* mRNA levels compared to the levels of 11 housekeeping genes and to the level of *ANG1* mRNA, at each of the 10 time points (Fig. 5*c*). Results show that *ANG2* is induced early by E2 in this cell line at 1 and 2 hr of treatment. The level still increases to the maximum at 12 hr and then starts decreasing.

These results strongly suggest that *ANG2* expression in response to estrogenic stimulation may have both primary and secondary components in different cell contexts.

DISCUSSION

Angiopoietins are important factors in the angiogenic process, but their role in tumor angiogenesis is not yet elucidated. Angiopoietins and VEGFs seem to play coordinated roles in the development of new vessels to support tumor growth.[44] We demonstrate that angiopoietins 1 and 2 are both expressed at a measurable level in the majority of breast cancers and that angiopoietin 2 shows a clear-cut association with tumor aggressiveness. It is remarkable, indeed, that given the small number of cases included in our study, *ANG2* mRNA level was a significant predictor of recurrence and death in multivariate analysis and in the node-negative subgroup as well. The density of blood vessels has already been reported as a prognostic factor,[19–25] and the fact that *ANG2* correlates with vessel density further confirms this finding. The prognostic value of *ANG2* mRNA levels was already demonstrated in gastric carcinoma.[33] Confirmation of *ANG2* value in another kind of cancer, especially in the case of node-negative breast cancer, where formulation of prognosis is particularly difficult, could have a relevant clinical impact.

The fact that *ANG2* associates with tumor aggressiveness, whereas *ANG1* does not, fits with our current understanding of how the network of these factors work in angiogenesis. In the presence of *VEGF*, in fact, *ANG2* action is necessary to induce angiogenesis, since it appears to be the primary factor to destabilize formed vessels and initiate sprouting.[3,12,18,45,46] Since *VEGF* and the Angiopoietins play complementary and coordinated roles in the development of new blood vessels[12,15,17,45,46] and several articles reported that *VEGF-C* has an important role in lymph-node metastasis of breast cancer,[47,48] it could be very interesting to see whether *VEGF* and/or *VEGF-C* expression is present in tumors overexpressing *ANG2*. RT-PCR analysis of *VEGF* and *VEGF-C* was performed on a small subset of tumors, where an aliquot of the same RNA employed for angiopoietin analysis was available. Results of this analysis, summarized in Table II, show that even though *ANG2* mean values are definitely higher in *VEGF*+ and *VEGF-C*+ tumors, no statistically significant correlation can be found among these factors. Due to the small number of cases examined, this result should be considered as preliminary and the importance of this issue deserves further studies.

Based on immunohistochemical analysis, no or very low expression of Ang2 is present in normal mammary tissues. However, *ANG2* mRNA levels comparable to those measured in breast carcinoma-derived cell lines were found in the nontumorigenic MCF10A cells (Fig. 4) and in other nontumorigenic cell lines (not shown). This is not surprising, since during the normal development of the mammary gland during pregnancy, there is a substantial angiogenesis and it is possible that epithelial cells provide signals sustaining this activity. Unfortunately, it is quite difficult to define a real normal counterpart for breast cancer cells, since the histology and endocrinology of the mammary gland changes continuously during life, so that whether *ANG2* overexpression is a tumor-specific event remains an open issue.

Expression of *ANG2* is present in both tumor cells and in tumor endothelium.[30,32] While its expression in endothelial cells is a well-established notion,[12,49,50] we do not have enough data to discriminate whether tumor cell ability to produce Ang2 may be the key factor in determining a more aggressive phenotype. Clearly, if tumor cells can produce Ang2 on their own, this could be quite important to allow metastatic growth through direct vascular remodeling at that site. To answer these questions, a larger study should be devised to compare immunohistochemical patterns with clinical data. Also, it may be important to determine whether *ANG2* expression is a stable phenotype, *i.e.,* if it is conserved in lymph node and distant metastases. Our data clearly demonstrate that *ANG2* expression is maintained in established breast carcinoma cell lines. It is not known whether upregulation results from transcriptional activation or by genomic rearrangements in tumors. Evidence that *ANG2* transcription is widely regulated by several factors, among which are growth-regulatory agents, hyperthermia and hypoxia, has been recently demonstrated.[30,51] In particular, our experiments suggest that estrogen strongly increases *ANG2* mRNA levels in breast cancer cells, at least transiently and with different kinetics in different cell lines, perhaps suggesting that the regulation of *ANG2* gene responds to different pathways. Stimulation of *ANG2* expression by estrogen *in vivo* was recently reported,[52] and this could confirm our data. On the other side, no correlation was found between *ANG2* and ER expression levels in tumors (Table I). It is therefore very likely that estrogen stimulation is only one of the possible mechanisms sustaining *ANG2* expression and that other pathways are operative in ER-negative tumors overexpressing *ANG2*. Inspection of the 5′-flanking region of the *ANG2* gene on chromosome 8 reveals the presence of several potential regulatory elements among which are some estrogen-regulated transcription factors, such as *c-MYB* and *AP-1*. However, no functional data on *ANG2* promoter are available to our knowledge. The possible relevance of our *in vitro* observation on estrogen effects deserves further investigations.

An additional important point is whether the expression of *ANG2* by tumor cells should contribute an autocrine signal for tumor growth. Expression of the angiopoietin receptor Tie2/Tek is more commonly found in endothelial cells, however, recently Currie *et al.* demonstrated the expression of Tie2 in breast tumors;[34] this information, together with our results, may suggest a possible autocrine and paracrine pathway in mammary tumor cells.

The expression of *ANG1* and *ANG2* mRNA (together with *ANG4*) in breast cancer tissues was reported by others,[34] but no correlation was found between lymph node involvement and *ANG2* expression, as measured by common RT-PCR followed by ethidium bromide staining evaluation. In our study, the use of the more quantitative real-time PCR method allowed us to consider

TABLE II – CORRELATION OF *ANG2* MRNA LEVELS WITH *VEGF* AND *VEGF-C* MRNA EXPRESSION IN A SUBSET OF PRIMARY BREAST CANCER (n = 14)

		Tumors with high *ANG2* mRNA/n	*ANG2* mRNA mean[1]	*ANG2* mRNA range[1]	p^2
VEGF	+	6/9	15.03	0.95–84.11	NS[2]
	−	2/5	3.28	1.24–5.87	
VEGF-C	+	6/10	13.66	0.95–84.11	NS
	−	2/4	3.80	1.26–5.87	

[1]Copies/μg total RNA.–[2]Student's t and Mann-Whitney tests.–[3]NS, not statistically significant.

ANG2 expression level as a continuous variable, and the level of expression displayed association with lymph node invasion. In addition, by using real-time RT-PCR, we found measurable levels of *ANG2* mRNA in all the samples, while the quoted study reported as positive only 25 out of 48 cases. Another study[53] reported expression of *ANG1* in only 3 out of 21 laser-capture microdissected breast tumors, concluding that this gene is rarely expressed *in vivo*. Again, we found measurable levels of *ANG1* mRNA in all tumor tissues examined, though displaying no correlation with clinicopathologic parameters. One possible explanation is that *ANG1* is more commonly expressed in tissue components other than tumor cells. Since our samples were microdissected but not selected by laser-capture, this may explain the discrepancy. Since Ang1 and Ang2 compete for the Tie2 receptor,[49] it is clear that the balance between these 2 factors is particularly important to determine biologic responses. Thus, a reduced level of Angiopoietin-1 may contribute to the neoangiogenetic effect of Angiopoietin-2 expression. Indeed, if tested using the Kaplan-Meier analysis, the *ANG2/ANG1* ratio shows a significant association with shorter OS and DFS (p, respectively, 0.0005 and 0.0001), suggesting that the loss of *ANG1* expression in tumor cells, together with the acquisition of *ANG2* expression, may contribute to tumor aggressiveness.

Breast cancer is a heterogeneous disease whose clinical outcome encompasses a wide spectrum of possibilities from definitive cure to early death. Available prognostic indicators are not accurate: despite axillary nodal status being the single most important prognostic factor, up to 30% of node-negative patients eventually relapse. Thus, there is a stringent need for additional markers; if larger and carefully designed studies will confirm our preliminary data, *ANG2* expression holds the promise to be one of such markers, given the strong biologic rationale supporting this hypothesis. In addition, new therapeutic options could derive from the blockade of *ANG2* expression as a systemic treatment both in the adjuvant and in the metastatic settings.

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