Clinical implications of expression of ETS-1 in relation to angiogenesis in ovarian cancers

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ETS-1 has been identified as a transcription factor involved in tumor angiogenesis, which is essential for the growth, invasion, and metastasis of solid tumors. This result prompted us to study whether ETS-1 works as an angiogenic mediator in ovarian cancers. Immunohistochemical staining revealed that ETS-1 was expressed in vascular endothelial cells and in cancer cells of ovarian cancers. There was a significant correlation between microvessel counts and both ETS-1 histoscores and ets-1 mRNA levels in ovarian cancers. Both ETS-1 histoscores and ets-1 mRNA levels increased with the progression of ovarian cancers. Furthermore, the 24-month survival rate of 30 patients with high ets-1 (high ETS-1 histoscores and high ets-1 mRNA levels) was 30%, while that of 30 other patients with low ets-1 (low ETS-1 histoscores and ets-1 mRNA levels) was 70%. There was a significant difference between the 24-month survival rates of the 30 patients with high ets-1 and the 30 with low ets-1. This indicates that ETS-1 might act as an angiogenic mediator in, and be a prognostic factor for, ovarian cancers. (Cancer Sci 2003; 94: 769–773)

Angiogenesis is essential for development, growth and progression of solid tumors.[1)] The angiogenic factors vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), platelet-derived endothelial cell growth factor (PD-ECGF; identical with thymidine phosphorylase (TP)), and interleukin (IL)-8 modulate angiogenesis in ovarian cancers.[2–5)] bFGF was found to be expressed in both cancer and interstitial cells of ovarian cancers, and its levels correlated with clinical stage, but not obviously with the patients' prognosis.[2)] PD-ECGF was dominantly expressed in interstitial cells of ovarian cancers, and a high level correlated with poor prognosis.[3)] VEGF, particularly $VEGF_{165}$ isomer, was dominantly expressed in cancer cells of primary ovarian cancers, and its level correlated with the patients' prognosis, regardless of histopathological findings.[4)] Furthermore, patients with higher VEGF levels in peritoneal metastatic lesions than in the primary tumors had poorer prognosis.[5)]

During angiogenesis, ETS-1 is strongly expressed in vascular endothelial cells and in the adjacent interstitial cells.[6)] Once angiogenesis has ended, ETS-1 expression is down-regulated.[7, 8)] The representative angiogenic factors VEGF and bFGF immediately induce ETS-1 expression in the early stage of angiogenesis, while the inhibition of ETS-1 expression leads to suppression of angiogenesis.[9, 10)] The proteases urokinase type-plasminogen activator (u-PA), matrix metalloprotease (MMP)-1, MMP-3 and MMP-9 have a conserved ETS-binding motif, and transcription factor ETS-1 converts vascular endothelial cells to angiogenic phenotypes by inducing expression of *u-PA, MMP-1, MMP-3 and MMP-9 and integrinβ3* genes.[11, 12)]

Ets-1 is expressed in various gastric, pancreatic, esophageal, and hepatocellular and cholangiocellular carcinomas and in thyroid and astrocytic tumors, and acts as a proto-oncogene.[13–19)] ETS-1 is up-regulated and involved in the overexpression of MMP-7 in hepatocellular carcinoma cells,[20)] and positively regulates the expression of u-PA in breast cancer, glioma, astrocytoma and meningioma cells, being associated with invasive potential and phenotypes.[21–24)] ETS-1 expression is induced by bFGF in glioma cells, in which it is related to invasive potential,[22)] and by VEGF in astrocytomas, in which it is related to angiogenesis.[25)] Furthermore, overexpression of ETS-1 is recognized as angiogenic in gastric and oral squamous cell carcinomas.[26, 27)] ETS-1 acts as an angiogenic mediator in uterine cervical and endometrial cancers.[28, 29)] These findings prompted us to study whether the proto-oncogene *ets-1* acts as an angiogenic mediator in ovarian cancers.

Materials and Methods

Patients. Prior, informed consent for the following studies was obtained from all patients and the Research Committee for Human Subjects, Gifu University School of Medicine. Sixty patients with ovarian cancers (stage I, 15 cases; stage II, 25 cases; stage III, 20 cases; and mucinous cystadenocarcinoma, 16 cases; serous cystadenocarcinoma, 14 cases; endometrioid carcinoma, 12 cases; serous papillary cystadenocarcinoma, 10 cases; clear cell carcinoma, 8 cases), ranging from 28 to 87 years of age, underwent curative resection, which produced macroscopically disease-free status, at the Department of Obstetrics and Gynecology, Gifu University School of Medicine, between October 1995 and December 1999. Prognosis was analyzed in relation to 24-month survival rate. None of the patients had received any therapy before the ovarian cancer tissue was taken. A part of each tissue was snap-frozen in liquid nitrogen for determination of the levels of ets-1 mRNA, and neighboring parts were subjected to histopathological study, including immunohistochemical staining for ETS-1 and factor VIII-related antigen. The clinical stage of ovarian cancers was determined according to the International Federation of Obstetrics and Gynecology (FIGO) classification.[30)]

Preparation of internal standard recombinant RNA for competitive RT-PCR-Southern blot analysis.[28, 29)] Deoxynucleic acid construction of the internal standard was done by PCR from a *Bam*HI/*Eco*RI-ligated fragment of V-erbB (Clontech Laboratories, Palo Alto, CA) with two sets of oligonucleotide primer sequences. The sequences of the first set of primers for ets-1 mRNA (MIMIC ets-1-5′ and MIMIC ets-1-3′) in the first PCR were as follows: MIMIC ets-1-5′, 5′-ATGGAGTCAACCCAGCCTATCGCAAGTGAAATCTCCTCCG-3′; MIMIC ets-1-3′, 5′-CCATGCACATGTTGTCTGGG-TCTGTCAATGCAGTTTGTA-3′.[31, 32)] The sequences of the second set of primers for ets-1 mRNA (MIMIC ets-1-P and ets-1-3′) in the secondary PCR were as follows: MIMIC ets-1-P, 5′-TAATACGACTCACTATAGG-ATGGAGTCAACCCAGCCTAT-3′; ets-1-3′, 5′-CCATGCACATGTTGTCTGGG-3′. The first and second PCRs were carried out as previously described.[28, 29)] The second PCR products were transcribed using T7 RNA polymerase (Gibco BRL, Gaithersberg, MD), and the amount of transcribed internal marker was calculated as previously described.[28, 29)]

Competitive RT-PCR-Southern blot analysis. Total RNA was isolated from the tissues by means of the acid guanidium thiocyan-

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ate-phenol-chloroform extraction method.[33] To obtain a standard curve each time, the total RNA (3 μg) and a series of diluted recombinant RNA for ets-1 mRNA (1 to 100 fmol) were reverse-transcribed. The sequences of primers used to amplify the *ets-1* gene (ets-1-5′ and ets-1-3′) were as follows: ets-1-5′, 5′-ATGGAGTCAACCCAGCCTAT-3′ (exon 5); ets-1-3′, 5′-CCATGCACATGTTGTCTGGG-3′ (exon 6). Competitive PCR was carried out as previously described.[28, 29] In the competitive RT-PCR-Southern blot analysis for ets-1 mRNA, only the two predicted sizes of DNA fragment hybridized with the biotinylated ets-1-5′ probe, allowing quantitation as previously described.[28, 29] In the negative control, no ets-1 mRNA was detected without reverse transcription in 30 cycles of PCR. The levels of ets-1 mRNA were determined using a standard curve and serial dilutions of rcRNA in competitive RT-PCR-Southern blot analyses as shown in Fig. 1.

Immunohistochemistry. Sections (4 μm) of formalin-fixed, paraffin-embedded ovarian cancer tissues were cut with a microtome and dried overnight at 37°C on a silanized slide (Dako, Carpinteria, CA). Samples were deparaffinized in xylene at room temperature for 80 min and washed with a graded ethanol/water mixture and then with distilled water. The samples for ETS-1 were soaked in a citrate buffer, and then microwaved at 100°C for 10 min, and those for factor VIII-related antigen were treated with 0.3 μg/ml trypsin in phosphate buffer at room temperature for 20 min. The protocol for a DAKO LSAB2 Kit, Peroxidase (Dako) was followed for each sample. In the described procedures, rabbit anti-human ETS-1 (C-20, Santa Cruz Biotechnology, Santa Cruz, CA), and rabbit anti-factor VIII-related antigen (Zymed, San Francisco, CA) were used at dilutions of 1:2000 and 1:2, respectively, as the first antibodies. The addition of the first antibody, rabbit anti-human ETS-1 or rabbit anti-factor VIII-related antigen, was omitted in the protocols for negative controls of ETS-1 or factor VIII-related antigen, respectively.

Vessels were counted in the five highest-density areas at 200× magnification (using a combination of 20× objective and 10× ocular, 0.785 mm^2 per field). Microvessel counts were expressed as the mean number of vessels in these areas.[34] Microvessel density was evaluated from microvessel counts.

The results of immunohistochemical staining for ETS-1 were semiquantitatively evaluated as described by McCarty *et al.*[35] Each stained section was given a histochemical score (histoscore, HS) calculated by applying the formula: $\sum(i+1)\times P_i$, in which i=cellular staining intensity (range 1 to 4; 0 indicates no staining) and P_i=percentage of stained cells.

Enzyme immunoassay for determination of human VEGF antigen. All steps were carried out at 4°C. Tissues of ovarian cancers (wet weight 10–20 mg) were homogenized in HG buffer (5

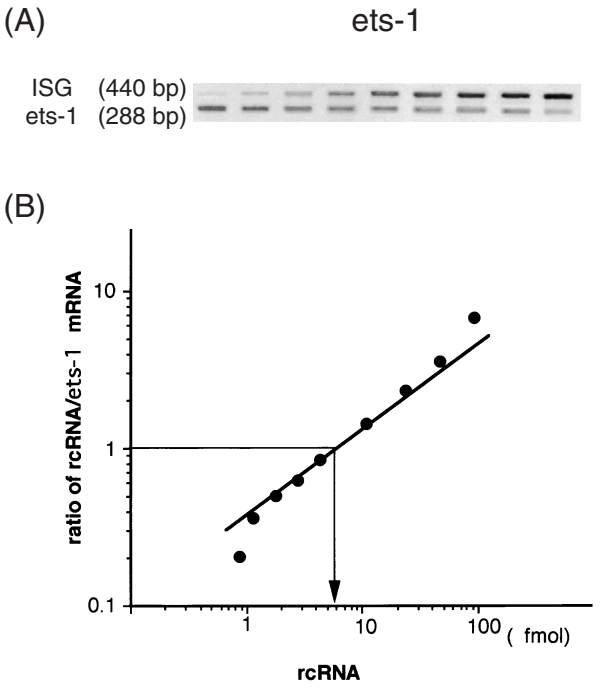

Fig. 1. Quantitative analysis of ets-1 mRNA by competitive RT-PCR-Southern blot analysis. The RT-PCR reactions containing *ets-1* gene-specific primers were carried out in the presence of total RNA and serially diluted internal standard recombinant RNA (rcRNA) in the range of 1 to 10^2 fmol for ets-1 mRNA. Southern blot analyses for the competitive RT-PCR are shown in panel (A). In panel (B), data are plotted to determine ets-1 mRNA levels as the log ratio of rcRNA/ets-1 mRNA total RNA isolated from the samples vs. log rcRNA.

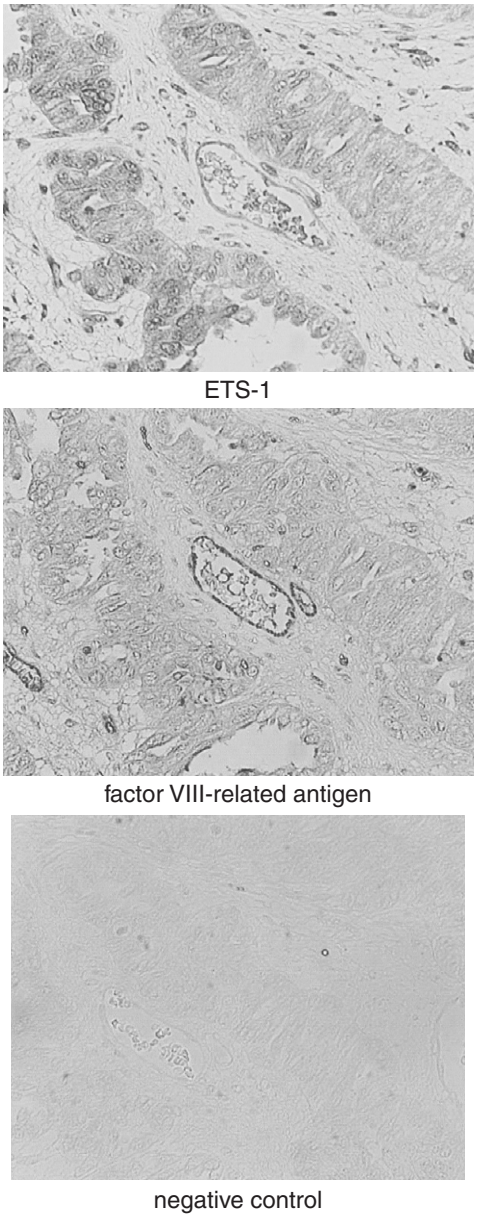

Fig. 2. Immunohistochemical staining for ETS-1 and factor VIII-related antigen in ovarian cancers (original magnification 200×). A case of ovarian cancer (serous papillary cystadenocarcinoma, stage III). Factor III-related antigen was clearly distributed in vascular endothelial cells. ETS-1 was distributed in the cancer cells and vascular endothelial cells.

m*M* Tris-HCl, pH 7.4, 5 m*M* NaCl, 1 m*M* $CaCl_2$, 2 m*M* $MgCl_2$, 2 m*M* dithiothreitol, 25 μg/ml aprotinin, and 25 μg/ml leupeptin) with a Polytron homogenizer (Kinematica, Luzern, Switzerland). This suspension was centrifuged in a microfuge at 10,000*g* for 3 min to obtain the supernatant. The protein concentration of samples was measured by the method of Bradford [36] to standardize VEGF antigen levels.

VEGF antigen levels in the samples were determined by a sandwich immunoassay using a Human VEGF Quantikine kit (R & D Systems, Minneapolis, MN). The levels of VEGF were standardized with the corresponding cellular protein concentrations.

Statistics. The levels of ets-1 mRNA and VEGF were measured in three parts taken from each tissue, and the assay of each sample was carried out in triplicate. The sample correlation coefficient was used for the comparisons (Figs. 3 and 5). Differences were considered significant when *P* was less than 0.05. The *t* test for two independent samples was used to compare the determinations (Fig. 4). Survival curves were calculated using the Kaplan-Meier method, and analyzed by means of the log-rank test (Fig. 6).

Results

Immunohistochemical staining for ETS-1 and factor VIII-related antigen in a representative case of serous papillary adenocarcinoma stage III is shown in Fig. 2. Factor VIII-related antigen was clearly distributed in vascular endothelial cells. ETS-1 was distributed in the cancer cells and vascular endothelial cells. There was a significant correlation between microvessel counts (MVC) and both ETS-1 histoscores ($P<0.001$) in the endothelial cells and ets-1 mRNA levels ($P<0.001$) in the tissues of ovarian cancers as shown in Fig. 3, regardless of histopathological type (data not shown).

Both ETS-1 histoscores and ets-1 mRNA levels increased with advancing disease stage of ovarian cancers, as shown in Fig. 4, regardless of histopathological type (data not shown). All patients underwent curative resection, which produced macroscopically disease-free status. The 24-month survival rate of the 30 patients with high ets-1 (cases with high ETS-1 histoscore, over 280; the same cases as those with high ets-1 mRNA levels, over 0.3 pmol rRNA/μg total RNA) was 30%, while that of the other 30 patients with low ets-1 (cases with low ETS-1 histoscore, below 280; with low ets-1 mRNA levels, below 0.3 pmol rRNA/μg total RNA) was 70%. The values of 280 in ETS-1 histoscore and 0.3 pmol rcRNA/μg total RNA in ets-1 mRNA level were the median values, and were therefore adopted to divide the patients into the two groups.

There was a significant correlation between VEGF levels and both ETS-1 histoscores ($P<0.001$) and ets-1 mRNA levels ($P<0.001$) in ovarian cancers, as shown in Fig. 5, regardless of histopathological type (data not shown).

There was a significant difference ($P<0.002$) between the 24-month survival rates of the 30 patients with high and the 30 with low ets-1 mRNA expression, as shown in Fig. 6.

Discussion

In our present study, ETS-1 was expressed in vascular endothelial cells and cancer cells of ovarian cancers. Furthermore, ETS-1 histoscores and ets-1 mRNA levels increased with advancing disease stage, and correlated with poor prognosis in ovarian cancers. Davidson *et al.* reported that ETS-1 was expressed in stromal cells and tumor cells of ovarian cancers, and that ETS-1 is thus a novel prognostic marker in advanced-stage ovarian cancers.[37] Interestingly, ETS-1 expression in both tumor ($P=0.018$) and stroma ($P=0.026$) correlated with poor survival. However, they did not explain how high ETS-1 expression in stromal cells might contribute to the progression of ovarian cancer or why it should be a prognostic marker.

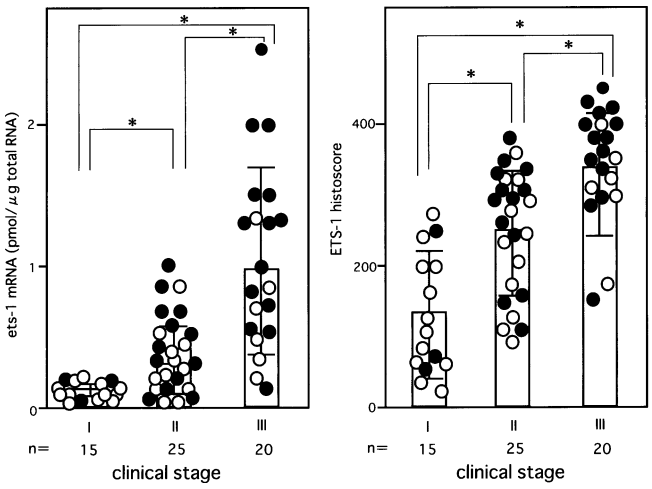

Fig. 4. ETS-1 histoscores and ets-1 mRNA levels in ovarian cancers classified according to clinical stages. Clinical stages of ovarian cancer were determined according to the FIGO classification. Each level is the mean of 9 determinations. Living and deceased patients are shown by ○ and ●, respectively. * $P<0.05$.

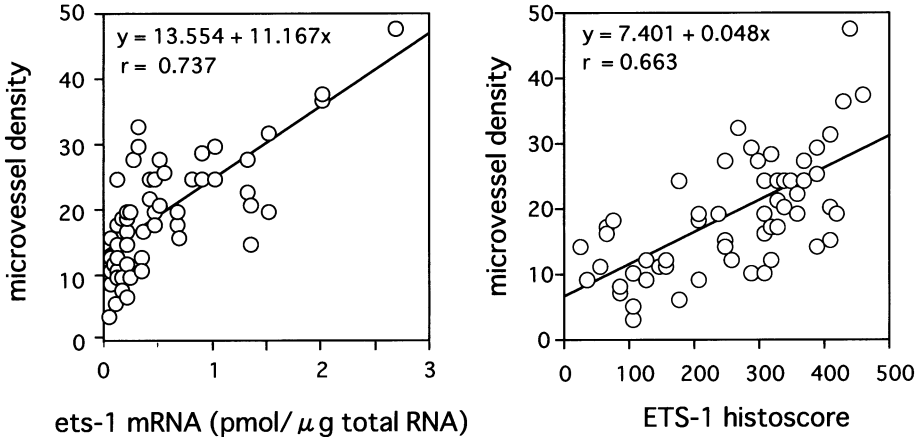

Fig. 3. Correlation between microvessel count and both ETS-1 histoscores and ets-1 mRNA levels in ovarian cancers.

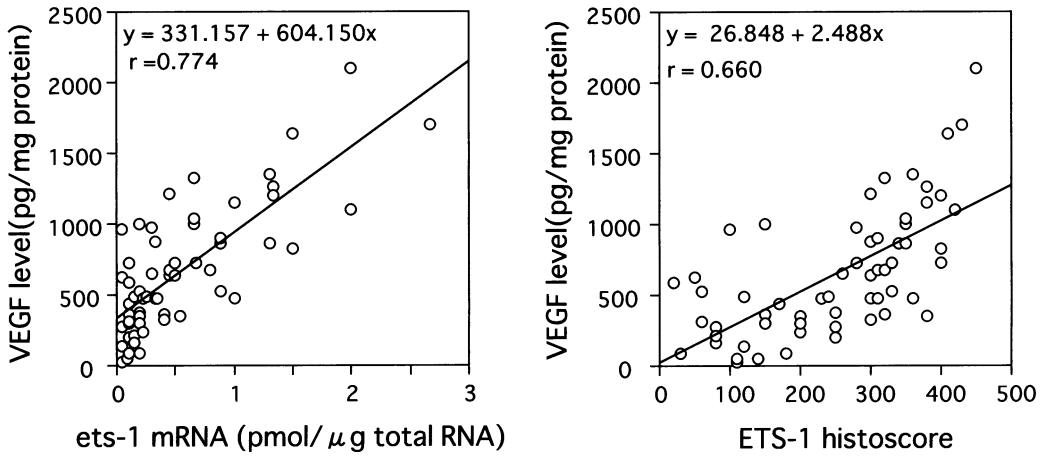

Fig. 5. Correlation between VEGF levels and both ETS-1 histoscores and ets-1 mRNA levels in ovarian cancers.

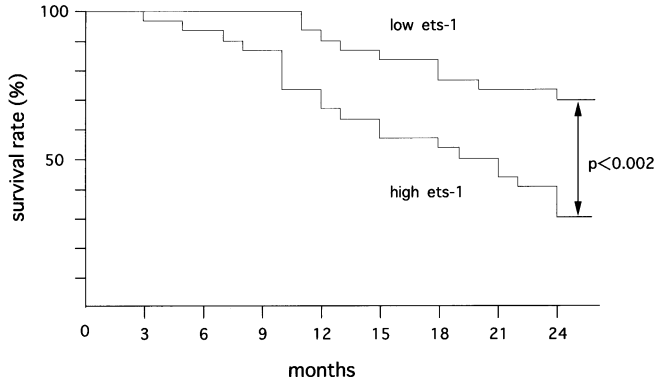

Fig. 6. Survival rate after curative resection for ovarian cancers. Patients' prognosis was analyzed in terms of the 24-month survival rate. High ets-1, cases with high ETS-1 histoscore (>280); these are the same as the cases with high ets-1 mRNA levels (>0.3 pmol rRNA/μg total RNA); n=30. Low ets-1, cases with low ETS-1 histoscore (<280); these are the same as the cases with low ets-1 mRNA levels (<0.3 pmol rRNA/μg total RNA); n=30.

Generally, distinct ETS-1 expression in vascular endothelial cells has been associated with accelerated angiogenesis.[6–8] ETS-1 might be activated as a direct angiogenic mediator for the initiation and maintenance stages of angiogenesis, and may be an excellent indicator of poor prognosis in ovarian cancers. It is probable that ETS-1 expressed in vascular endothelial cells directly induces angiogenesis, and ETS-1 expressed in cancer cells is likely to be indirectly associated with angiogenesis as a proto-oncogenic protein. Therefore, high ETS-1 expression in stromal cells, especially vascular endothelial cells, might contribute to the progression of ovarian cancers as an angiogenic mediator, and lead to a poor prognosis.

VEGF is the most important angiogenic factor for ovarian carcinomas, especially for promoting peritoneal dissemination,[4, 5] and VEGF has been shown to induce ETS-1 expression in vascular endothelial cell lines.[9] ETS-1 is a transcriptional factor for angiogenesis, and ets-1 is also a proto-oncogene that promotes various tumors.[13–19]

In the present study, ets-1 mRNA levels correlated with microvessel density observed in the immunohistochemical staining for factor VIII-related antigen. Furthermore, VEGF levels correlated with ETS-1 histoscores in the endothelial cells and ets-1 mRNA levels in the cancer tissues of all cases. VEGF is produced by cancer cells, and is transferred to the stromal cells, especially to the VEGF receptor sites on the endothelial cells, in ovarian cancers.[4] In the cancer cells, VEGF might induce ETS-1 expression and act as an oncogene. However, its function has not been elucidated yet. In the endothelial cells, VEGF from the cancer cells might induce ETS-1 expression, and ETS-1 might act as an angiogenic mediator in ovarian cancers. Further, even if the main angiogenic factors can be suppressed by therapeutic agents, angiogenesis might be suppressed only transiently, which could lead to temporary suppression of tumor growth and secondary spreading. In such a scenario, other angiogenic factors would be induced, and link to ETS-1 in the recruitment of alternative pathways of angiogenic activation, producing a kind of tolerance to angiogenic inhibitors. Therefore, suppression of the major angiogenic factors along with suppression of ETS-1 recruitment might be more effective as a tumor dormancy-inducing therapy than mere suppression of major angiogenic factors. A specific inhibitor for ETS-1, transdominant mutant ETS-1, has already been shown to act as a dominant negative molecule, and can be used as an efficient tool for angiogenic inhibition.[38]

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