perifosine were purchased from Selleck Chemicals (Houston, TX, USA). Antibodies against epidermal growth factor receptor (EGFR) (2232; Cell Signaling Technology, Inc, Beverly, MA, USA), phospho-EGFR (p-EGFR; Tyr 1069) (2234; Cell Signaling), PTEN (9552; Cell Signaling), Akt (9272; Cell Signaling), phospho-Akt (p-Akt; Ser473) (9271; Cell Signaling), mTOR (2972; Cell Signaling), phospho-mTOR (p-mTOR; Ser2448) (2971; Cell Signaling), S6 ribosomal protein (2212; Cell Signaling), phospho-S6 ribosomal protein (p–S6; Ser235/236) (2211; Cell Signaling), 4EBP1 (9452; Cell Signaling), phospho-4EBP1 (p-4EBP1; Ser65) (9451; Cell Signaling), E-cadherin (4065; Cell Signaling), Snail (ab17732; Abcam, Cambridge, UK), Twist (Twist2C1a; Bio Matrix Research, Chiba, Japan), and β-actin (4967; Cell Signaling) were also purchased.

Cell proliferation assays. Cell proliferation assays were performed using a Cell Counting Kit-8 assay (CCK-8; Dojindo, Kumamoto, Japan) according to the product protocol. Briefly, cells were plated into 96-well, flat-bottomed plates at $2–3 \times 10^3$ cells/180 μL/well. After overnight incubation, triplicate wells were treated with varying concentrations of everolimus ranging from 0.1 to 500 nM for 96 h. The existing medium was removed and replaced with 110 μL of fresh medium containing 10 μL of CCK-8 reagent and allowed to incubate for 4 h. Absorbance was measured for each well at a wavelength of 450 nm. The percent survival and IC_{50}-values were calculated as described previously.[26]

Western blotting. Cultured cells were washed with cold PBS and lysed in M-PER buffer (Pierce, Rockford, IL, USA). The protein concentration of the supernatant was measured using the bicinchoninic acid (BCA) protein assay (Pierce). The membrane was probed with the first antibody and then with horseradish-peroxidase-conjugated secondary antibody. The bands were visualized using enhanced chemiluminescence (ECL Plus Western Blotting Detection Kit; Amersham, Piscataway, NJ, USA).

Immunohistochemistry. Cells were cultured in chamber slides for 48 h. The cultured cells were washed with PBS and fixed with 100% ethanol. The slides were then treated with 3% hydrogen peroxide for 30 min. The slides were incubated with primary antibodies against cytokeratin (CK) 5/6 protein (1:40; Dako Cytomation, Glostrup, Denmark) for 60 min at room temperature. Immunoreactions were detected using the EnVison Plus system (Dako).

Fluorescent *in situ* hybridization analysis. All the cell lines were cultured in appropriate media supplemented with 10% FBS; the FISH analyses were outsourced to SRL (Tokyo, Japan).

DNA sequencing. Sequencing was performed to detect the following mutations: in *EGFR*,[27] deletions in exon 19 (del 19) and L858R in exon 21; in *PI3KCA*,[28,29] E542K and E545K in exon 9 and H1047R in exon 20; and in *AKT1*,[30] E17K in exon 4. Briefly, the total RNAs were extracted from each cell line using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. First-strand cDNA was synthesized from 1 μg of total RNA using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). The first-strand cDNA was amplified by PCR using specific primers for *EGFR*. Genomic DNA was extracted from each cell line using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany), and exon regions were amplified via PCR using specific primers for *PI3KCA* and *AKT1*. DNA sequencing of the PCR products was performed using the dideoxy chain termination method and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Small interfering RNA treatment. Individual small interfering RNA (siRNA) duplexes specific to human PTEN (Invitrogen, Carlsbad, CA, USA) or a control siRNA that does not target

any sequence in the human genome (non-target control; Invitrogen) was transfected into MDA-MB-231 and BT20 cells according to the product protocol. Briefly, for each well transfection, we prepared RNAi duplex–Lipofectamine RNAiMax Transfection Reagent (Invitrogen) complexes with final a concentration of 10 nM and diluted the cells in complete growth medium without antibiotics. We added 3×10^3 cells to each well with RNAi duplex–Lipofectamine RNAiMax complexes. After 24 h of incubation, the medium was removed and replaced with fresh medium with 10% FBS and antibiotics containing various concentrations of everolimus for the cell proliferation assay.

Transfection of wild-type EGFR. Constructs of wild-type EGFR (EGFRwt) and empty vectors were generously contributed by Dr Kazuto Nishio (Osaka, Japan). The pVSV-G vector (BD Biosciences Clontech, Mountain View, CA, USA) for the constitution of the viral envelope and the pQCXIX constructs were co-transfected into HEK293 cells (BD Biosciences Clontech) using a FuGENE6 transfection reagent (Roche Diagnostics, Basel, Switzerland). Forty-eight hours after transfection, the culture medium was collected, and the viral particles were concentrated by centrifugation. MDA-MB-231 and MDA-MB-436 target cells were infected using a virus-containing medium according to standard procedures and were used for the cell proliferation assay.

Xenograft studies. Experiments were performed in accordance with the United Kingdom Coordinating Committee on Cancer Research Guidelines for the welfare of animals in experimental neoplasia (second edition).

Suspensions of MDA-MB-231 and MDA-MB-468 cells (5×10^6) were injected subcutaneously into the backs of 5-week-old BALB/cAJcl-nu/nu mice (CLEA Japan, Tokyo, Japan). After 5 weeks (tumors > 120 mm^3), the mice were randomly allocated into groups of five animals to receive everolimus (10 mg/kg per day, three times per week) or the vehicle only by oral gavage for 3 weeks. The tumor diameter and body weight of each mouse were measured three times weekly. The tumor diameters were measured using calipers three times per week to evaluate the effects of treatment, and the tumor volume was determined using the following equation: tumor volume = $ab^2/2$ (mm^3) (where a is the largest diameter of the tumor and b is the shortest diameter). Day "x" denotes the day on which the effect of the drugs was estimated and day "0" denotes the first day of treatment. All the mice were killed on day 22 after measuring their tumors.

Results

Sensitivity to everolimus in TNBC cell lines. We screened nine TNBC cell lines for sensitivity to everolimus. The IC_{50} values for everolimus in the nine cell lines ranged from 0.7 nM to over 200 nM (Fig. 1). As also shown in Figure 1, everolimus effectively inhibited growth in five of the nine cell lines at an IC_{50} under 100 nM. Among them, MDA-MB-468, Hs578T, and BT549 were highly sensitive to everolimus, with an IC_{50} of around 1 nM. We examined the induction of apoptosis in everolimus-sensitive cell lines using three different assays. However, we did not observe any significant apoptosis events (data not shown). In addition, we examined the growth-inhibitory effect of GDC0914 (a PI3K inhibitor) and perifosine (an Akt inhibitor) using MDA-MB-468 and BT549, two everolimus-sensitive cell lines, and MDA-MB-231 and MDA-MB-157, two everolimus-resistant cell lines. We found no significant differences in sensitivity to either inhibitor between everolimus-sensitive cell lines and resistant cell lines with a sub-μM IC_{50} concentration (data not shown).

Baseline expressions of proteins in the mTOR cascade. We measured the protein expressions of PTEN, p-AKT, Akt,
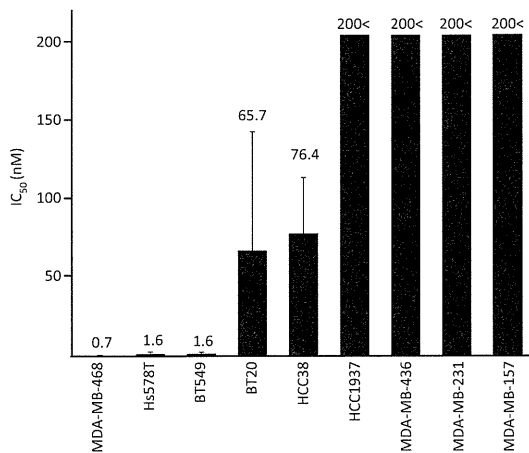
Fig. 1. IC_{50} values for everolimus of nine triple-negative breast cancer (TNBC) cell lines. Each cell line was treated with the indicated concentrations of everolimus for 96 h. Viable cell numbers were relatively quantified using the CCK-8 assay and were expressed as a percent of the untreated control.

p-mTOR, mTOR, p-S6, S6, p-4EBP1, and 4EBP1 using a western blot analysis in the nine TNBC cell lines (Fig. 2a). No differences in the expression levels of p-mTOR and mTOR, which are the targets of everolimus, were seen among the TNBC cell lines. As also shown in Figure 2(a), PTEN was not detected in five cell lines, among which MDA-MB-468, BT549, HCC1937, and MDA-MB-436 have been reported to harbor a somatic mutation of *PTEN*.[31] Among the five cell lines with a loss of PTEN, MDA-MB-468, BT549, and HCC38 were sensitive to everolimus; however, HCC1937 and MDA-MB-436 were resistant to everolimus.

mTOR cascade modulation by everolimus. To observe the effect of everolimus, we measured the protein expressions of p-S6 and S6 before and after everolimus treatment in MDA-MB-468 and BT549, two everolimus-sensitive cell lines, and MDA-MB-231 and MDA-MB-157, two everolimus-resistant cell lines. Equivalent reductions of p-S6 and S6 in response to only 0.5 nM of everolimus were observed in all four cell lines (Fig. 2b), indicating that everolimus acted on all the cell lines. We also measured the protein expressions of p-Akt and Akt and found an elevation in p-Akt after treatment with everolimus in BT549, MDA-MB-231, and MDA-MB-157 cells; however, this result was not observed in MDA-MB-468 cells.

Basal markers and cancer stem cell markers. Nielsen *et al.*[32] defined basal-like breast cancers as those showing a positive expression of EGFR or CK5/6 in TNBCs. In our results, EGFR was overexpressed in MDA-MB-468, Hs578T, BT549, and BT20 cells (Fig. 3a), while MDA-MB-468 and BT20 cells exhibited the gene amplification of *EGFR* (Fig. 3b). No mutations in the *EGFR* gene (del E746–A750, L858R) were detected in any of the cell lines (data not shown). CK5/6 was positive in MDA-MB-468, BT20, and HCC38 cells based on an immunocytochemical analysis (Fig. 3c). The status of basal-cell-like markers in the nine TNBC cell lines is shown in Table 1. According to the definition by Nielsen *et al.*,[32] we were able to categorize all five of the sensitive cell lines – MDA-MB-468, Hs578T, BT549, BT20, and HCC38 – as basal-like breast cancer. In contrast, the four resistant cell lines were not characterized as basal-like breast cancer. We also measured the expressions of the cancer stem cell marker proteins, E-cadherin, Snail, and Twist in all of the cell lines. E-cadherin was decreased in Hs578T, BT549, MDA-MB-436, MDA-MB-231, and MDA-MB-157. The expression of Snail gradually increased in the more resistant cells (HCC1937,

MDA-MB-436, MDA-MB-231, and MDA-MB-157). Twist was overexpressed in MDA-MB-436 and MDA-MB-157 cells. In summary, the resistant cell lines tended to show characteristics of cancer stem cells, with decreased E-cadherin expression and the increased expression of Snail or Twist.

Effect of PTEN knockdown or EGFR overexpression on everolimus sensitivity. We used siRNA oligonucleotides to silence the expression of PTEN in the BT20 and MDA-MB-231 cell lines to test whether PTEN expression confers everolimus sensitivity. Before the cell proliferation assay, we determined that the siRNA oligonucleotides against PTEN selectively reduced the mRNA expression levels by 80% or more after 48 h and that the PTEN protein levels were reduced after 72 h, compared with a nonspecific control siRNA. However, the sensitivity of these cells to everolimus did not change with the loss of PTEN expression. The results for MDA-MB-231 are shown in Figure 4(a).

We transfected construct expressing EGFRwt into the EGFR-silenced cell lines MDA-MB-231 and MDA-MB-436 to determine the effect of EGFR expression on everolimus sensitivity. However, the overexpression of EGFR did not affect the sensitivity to everolimus. The results for MDA-MB-231 are shown in Figure 4(b).

In vivo antitumor effects. To determine whether everolimus is also effective against basal-like breast cancer *in vivo*, the growth inhibitory effect was evaluated against MDA-MB-468, basal-like breast cancer cell line, and MDA-MB-231, non-basal-like breast cancer cell line, tumor xenografts. Everolimus treatment (10 mg/kg day, three times per week for 3 weeks) significantly suppressed the tumor volumes of the MDA-MB-468 xenografts, with T/C values of 38.3% (P = 0.016) on day 22 (Fig. 5a). On the other hand, everolimus treatment did not significantly suppress the tumor volumes of the MDA-MB-231 xenografts, with T/C values of 58.7% (P = 0.35) on day 22 (Fig. 5b). Body weight loss after treatment was not observed in the MDA-MB-468 and MDA-MB-231 xenograft groups (data not shown).

Discussion

Patients with TNBCs have relatively poor outcomes and cannot be treated with endocrine therapy or therapies targeted to HER2 receptors.[1] The lack of tailored therapies is problematic for the treatment of TNBCs, and the development of novel therapies is crucial. In this study, everolimus effectively inhibited growth in some TNBC cell lines with a sub-nM IC_{50} concentration *in vitro*. In previous reports, everolimus has shown limited growth-inhibitory activities against several human cancer cell lines, compared with TNBC cell lines.[33,34] Our results suggest that everolimus is a promising therapy for TNBCs.

The classification of TNBC subgroups is necessary for the future development of therapies. In this study, we found that TNBC cell lines classified as basal-like breast cancer were highly sensitive to everolimus, while cell lines characterized as cancer stem-cell-like were less sensitive to everolimus. Similar to the results of the *in vitro* assay, we found that treatment with everolimus significantly inhibited tumor growth in basal-like breast cancers *in vivo*. In a previous report, EGFR expression was associated with a poor prognosis and was a significant independent negative prognostic factor in a multivariate analysis.[32] Voduc *et al.*[35] reported that the risk of local and regional relapse in basal-like breast cancer was higher than those in other breast cancer subtypes. Our results suggest that everolimus is a promising therapy targeting basal-like TNBC.

Previous studies have suggested that the loss of PTEN may predict sensitivity to everolimus, since PTEN dysfunction leads to the activation of the PI3K/Akt/mTOR signaling
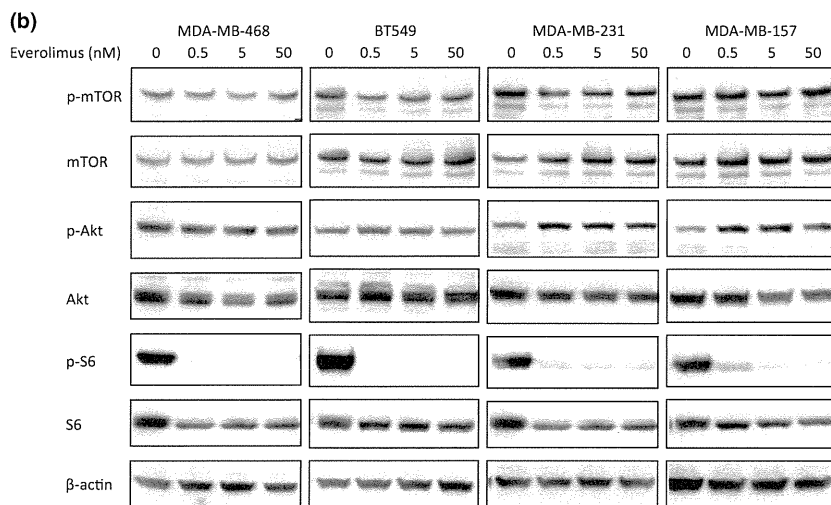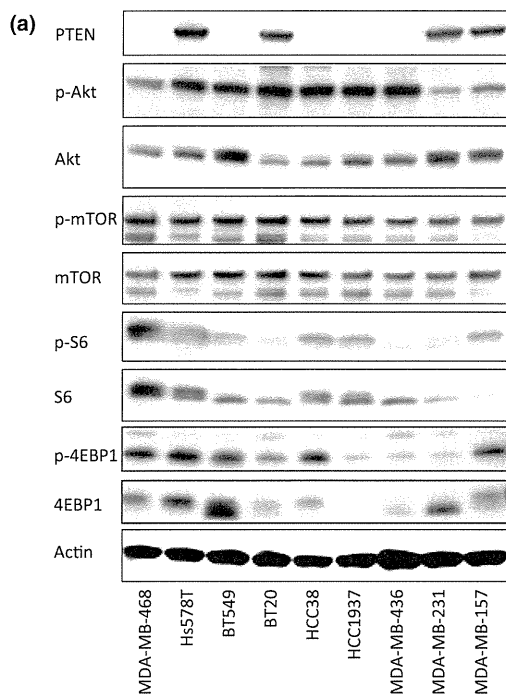
Fig. 2. Baseline expressions of mammalian target of rapamycin (mTOR) cascade proteins and mTOR cascade modulation by everolimus in nine triple-negative breast cancer (TNBC) cell lines. (a) Baseline protein expressions of PTEN, p-Akt, Akt, p-mTOR, mTOR, p-S6, S6, p-4EBP1, 4EBP1, and β-actin in nine TNBC cell lines. Ten micrograms of protein were prepared from the indicated cell lines at 60–70% confluence. The cell lines are arranged in decreasing order of sensitivity to everolimus, from left to right. β-Actin was used as a loading control. (b) Cells were untreated or treated with 0.5, 5, or 50 nM of everolimus for 1 h. Five micrograms of protein were prepared from the indicated cell lines. Equivalent reductions of p-S6 and S6 were observed with only 0.5 nM in the two sensitive cell lines (MDA-MB-468 and BT549) and the two resistant cell lines (MDA-MB-231 and MDA-MB-157).

pathway.[36,37] In the present study, three of the five cell lines that were sensitive to everolimus were PTEN-deficient cells, and the other two sensitive cell lines exhibited normal levels of PTEN protein expression. Two of the four everolimus-resistant cell lines were PTEN-deficient cells. Furthermore, in the siRNA experiment, although silencing the expression of PTEN activated Akt in BT20 and MDA-MB-231 cells, the sensitivities of these cell lines to everolimus did not change. Our results indicate that PTEN deficiency does not predict the response to everolimus in TNBCs. Furthermore, in a clinical trial with glioblastoma patients, no correlation between PTEN deficiency and the response to everolimus was observed.[38] Thus, these results suggest that mTOR was not controlled only by Akt, but also by multiple factors and signaling pathways. Furthermore, the role of PTEN in the response to everolimus may differ according to the type of cancer.

In this study, the expression of EGFR was correlated with the sensitivity to everolimus, and we considered the possibility that EGFR may be a key molecule in determining efficacy. EGFR overexpression in breast cancer has been reported in approximately 20–30% of all cases.[39,40] In TNBCs, EGFR expression was reported in 41–57% of cases and EGFR amplification was reported in 18%.[32,41] However, we found that EGFR transfection did not affect the sensitivity to everolimus, suggesting that there is another cascade that affects everolimus sensitivity. We observed an elevation in phosphor EGFR after everolimus treatment in a sensitive cell line, suggesting that a feedback mechanism might influence the sensitivity to everolimus. We examined the induction of apoptosis using three different assays; however, we did not observe any significant apoptosis events. The difference in the sensitivity of the TNBCs can likely be explained by some mechanism of action other than apoptosis. Further studies are needed to clarify these mechanisms and to elucidate the mechanism responsible for the sensitivity to everolimus in TNBCs. Furthermore, we suggest that combination strategies including mTOR inhibitor and PI3K or MEK inhibitor are needed in future clinical trials to overcome the multiple cascades or compensatory feedback systems resulting in cell survival.
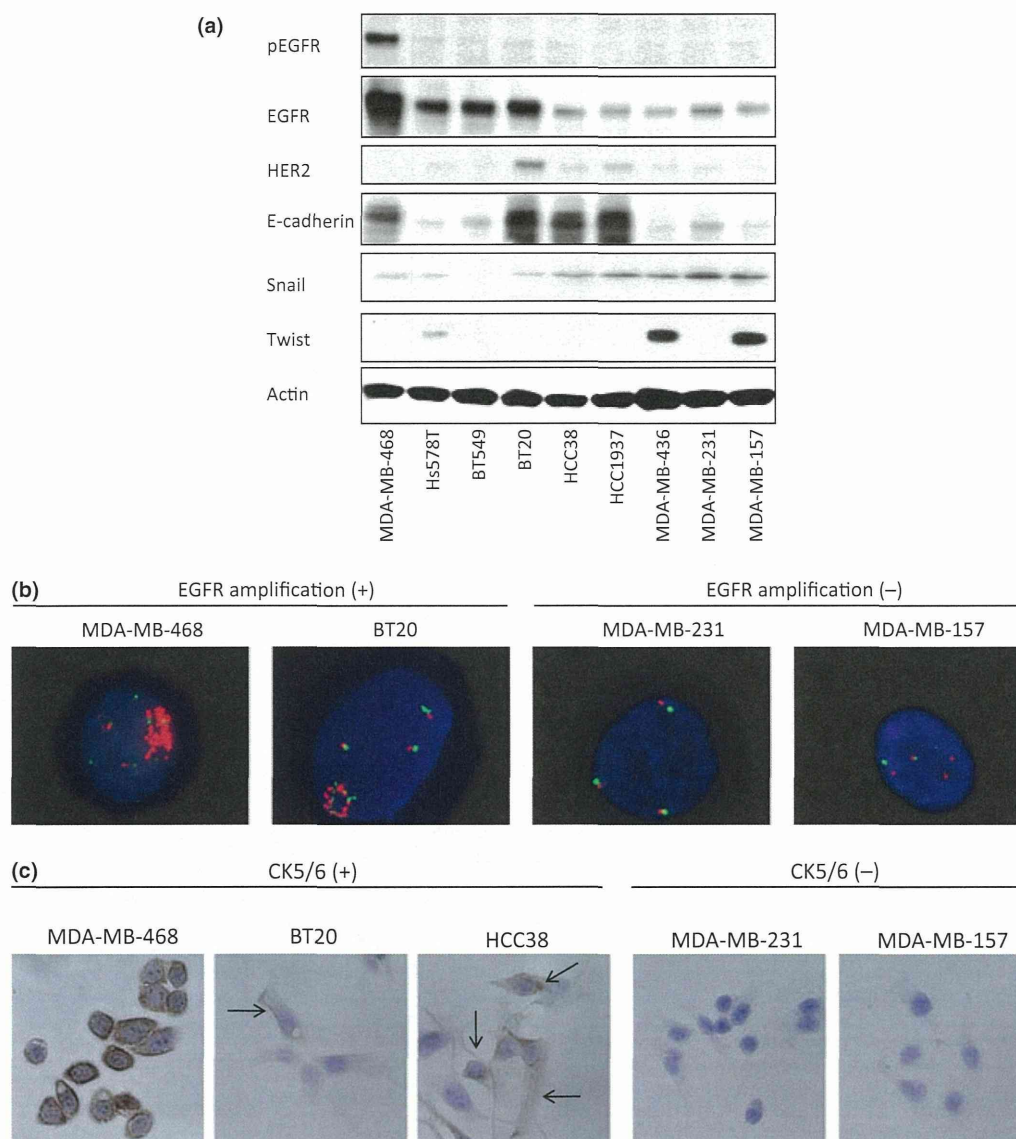
Fig. 3. Determination of breast cancer subtypes using basal markers and stem cell-like characteristics. (a) Protein expressions of p-EGFR, EGFR, HER2, E-cadherin, Snail, Twist, and β-actin in nine triple-negative breast cancer (TNBC) cell lines. Ten micrograms of protein were prepared from the indicated cell lines at 60–70% confluence. The cell lines are arranged in order of decreasing sensitivity to everolimus, from left to right. β-Actin was used as a loading control. (b) Epidermal growth factor receptor (EGFR) gene fluorescence *in situ* hybridization (FISH) analysis. MDA-MB-468 and BT20 showed the gene amplification of EGFR. The other seven TNBC cell lines did not exhibit the gene amplification of EGFR. Two positive cell lines and two negative cell lines are shown. (c) Immunohistochemical analysis of CK5/6. MDA-MB-468, BT20, and HCC38 were positive for CK5/6, and the other six cell lines were negative. Three positive cell lines and two negative cell lines are shown. Cell membranes were stained by the CK 5/6 antibody in all MDA-MB-468 cells and in some BT20 and HCC38 cells (indicated by arrows).

Table 1. The status of basal-cell-like markers in the triple-negative breast cancer (TNBC) cell lines

	MDA-MB-468	Hs578t	BT549	BT20	HCC38	HCC1937	MDA-MB-436	MDA-MB-231	MDA-MB-157
EGFR protein	+++	++	++	++	±	±	±	±	±
EGFR amplification	+++	−	−	++	−	−	−	−	−
EGFR mutation†	−	−	−	−	−	−	−	−	−
CK5/6 protein	++	−	−	+	+	−	−	−	−
PIK3CA mutation‡	−	−	−	H1047R	−	−	−	−	−
AKT1 mutation§	−	−	−	−	−	−	−	−	−

†*EGFR* mutations: deletions in exon 19 (del 19) and L858R in exon 21. ‡*PI3KCA* mutations: E542K and E545K in exon 9 and H1047R in exon 20. §*AKT1* mutation: E17K in exon 4.
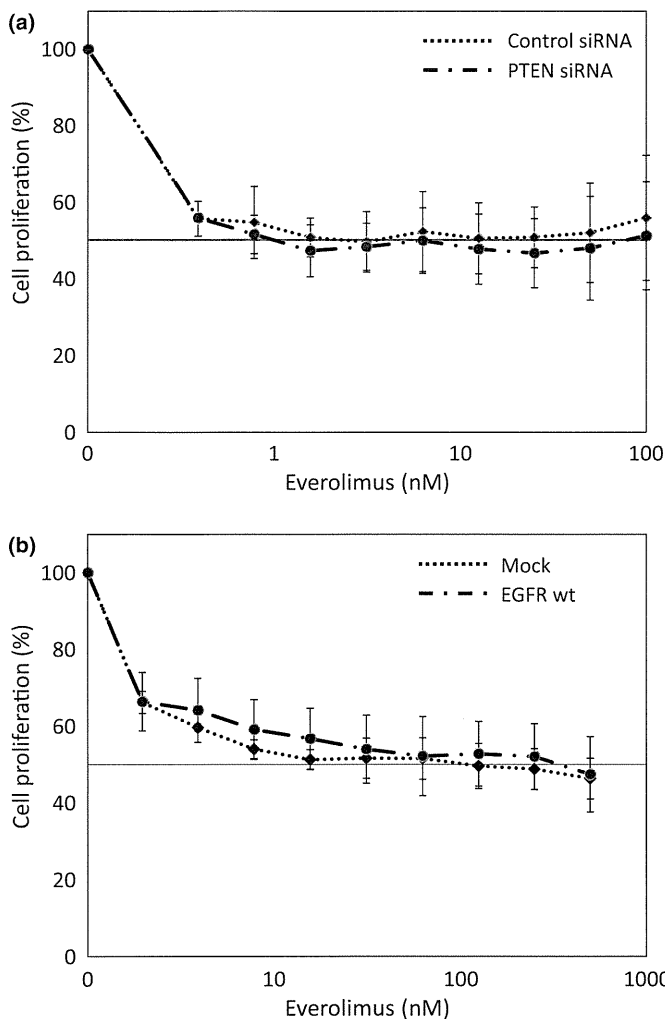
Fig. 4. Effect of PTEN or epidermal growth factor receptor (EGFR) modulation on everolimus sensitivity. (a) MDA-MB-231 and BT20 cells were transfected with siRNA specific to human PTEN or nonspecific control siRNA. After 24 h, the cells were treated with the indicated concentrations of everolimus for 72 h. The results for MDA-MB-231 are shown. (b) MDA-MB-231 and MDA-MB-436 cells were transfected with retrovirus containing either an empty vector or an EGFRwt vector and then were treated with the indicated concentrations of everolimus for 96 h. The results for MDA-MB-231 are shown.
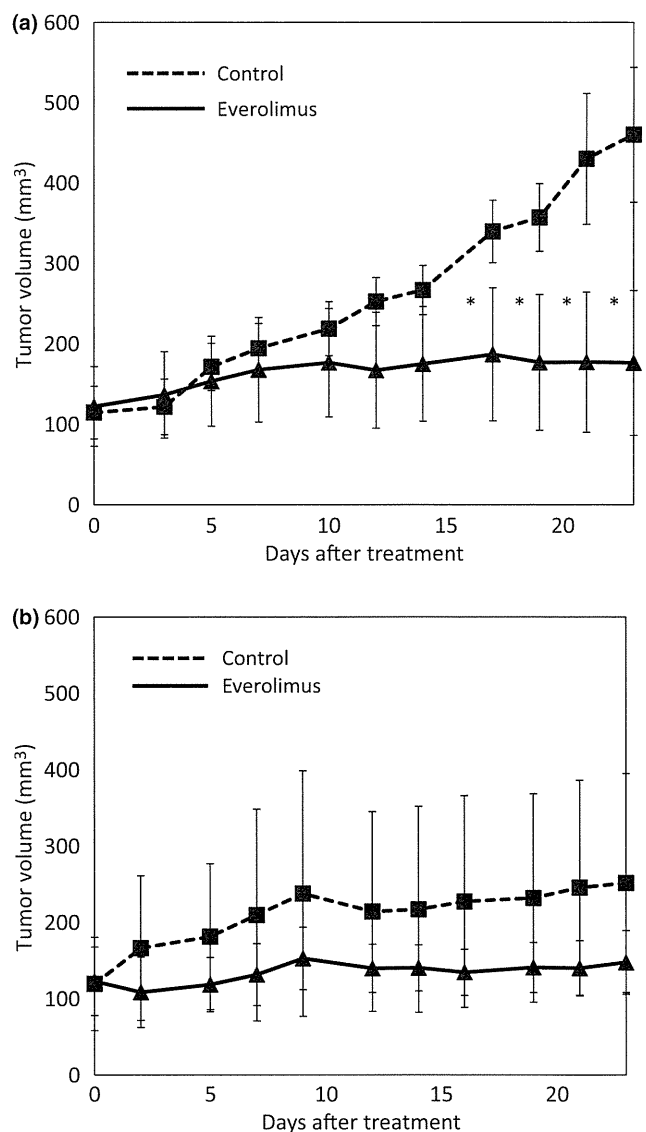

Fig. 5. Effect of RAD001 on the growth of breast cancer cell lines *in vivo*. Athymic nude mice were inoculated with MDA-MB-468 cells (a) or MDA-MB-231 cells (b). When the tumors reached an average size of 120 mm^3, mice were treated with placebo or 10 mg/kg per day RAD001, three times per week for 3 weeks. The tumors were measured twice weekly and tumor size was averaged for each treatment group. Points, mean; bars, standard deviation (SD); *P < 0.05, significantly different from placebo-treated mice.

Recent reports have indicated that the emergence of cancer stem cells occurs, in part, as a result of the epithelial-mesenchymal transition (EMT).[42,43] The EMT is characterized by a decrease in epithelial-specific gene expression, including E-cadherin, and a gain in mesenchymal-specific gene expression, including *twist* and *snail*.[14,15] Our data shows that cancer stem cell-positive TNBC cells tend to be resistant to everolimus. These EMT-rich TNBCs do not respond to traditional cytotoxic drugs or targeted therapies that act on signal transduction. Thus, other therapeutic strategies against these TNBCs, such as stem cell- or EMT-targeted drugs, are urgently needed.

This study suggests that everolimus is a promising agent for the treatment of TNBCs, especially basal-like breast cancers. Basal markers (EGFR and CK5/6) or cancer stem cell markers (E-cadherin, snail, or twist) may be predictive markers of the response to everolimus in TNBCs.

Acknowledgments

Disclosure Statement

The authors have no conflicts of interest.

References

1 Dent R, Trudeau M, Pritchard KI *et al.* Triple-negative breast cancer: clinical features and patterns of recurrence. *Clin Cancer Res* 2007; **13**: 4429 –34.
2 Liedtke C, Mazouni C, Hess KR *et al.* Response to neoadjuvant therapy and long-term survival in patients with triple-negative breast cancer. *J Clin ncol* 2008; **26**: 1275–81.
3 Thike AA, Cheok PY, Jara-Lazaro AR, Tan B, Tan P, Tan PH. Triple-negative breast cancer: clinicopathological characteristics and relationship with basal-like breast cancer. *Mod Pathol* 2010; **23**: 123–33.
4 Colleoni M, Cole BF, Viale G *et al.* Classical cyclophosphamide, methotrexate, and fluorouracil chemotherapy is more effective in triple-negative, node-negative breast cancer: results from two randomized trials of adjuvant chemoendocrine therapy for node-negative breast cancer. *J Clin Oncol* 2010; **28**: 2966–73.
5 Foulkes WD, Stefansson IM, Chappuis PO *et al.* Germline BRCA1 mutations and a basal epithelial phenotype in breast cancer. *J Natl Cancer Inst* 2003; **95**: 1482–5.
6 Hennessy BT, Giordano S, Broglio K *et al.* Biphasic metaplastic sarcomatoid carcinoma of the breast. *Ann Oncol* 2006; **17**: 605–13.
7 Perou CM, Sorlie T, Eisen MB *et al.* Molecular portraits of human breast tumours. *Nature* 2000; **406**: 747–52.
8 Sorlie T, Perou CM, Tibshirani R *et al.* Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci USA* 2001; **98**: 10869–74.
9 van't Veer LJ, Dai H, van de Vijver MJ *et al.* Gene expression profiling predicts clinical outcome of breast cancer. *Nature* 2002; **415**: 530–6.
10 Sotiriou C, Neo SY, McShane LM *et al.* Breast cancer classification and prognosis based on gene expression profiles from a population-based study. *Proc Natl Acad Sci USA* 2003; **100**: 10393–8.
11 Herschkowitz JI, Simin K, Weigman VJ *et al.* Identification of conserved gene expression features between murine mammary carcinoma models and human breast tumors. *Genome Biol* 2007; **8**: R76.
12 Hennessy BT, Gonzalez-Angulo AM, Stemke-Hale K *et al.* Characterization of a naturally occurring breast cancer subset enriched in epithelial-to-mesenchymal transition and stem cell characteristics. *Cancer Res* 2009; **69**: 4116–24.
13 Taube JH, Herschkowitz JI, Komurov K *et al.* Core epithelial-to-mesenchymal transition interactome gene-expression signature is associated with claudin-low and metaplastic breast cancer subtypes. *Proc Natl Acad Sci USA* 2010; **107**: 15449–54.
14 Cano A, Perez-Moreno MA, Rodrigo I *et al.* The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nat Cell Biol* 2000; **2**: 76–83.
15 Yang J, Mani SA, Donaher JL *et al.* Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. *Cell* 2004; **117**: 927–39.
16 Lu CH, Wyszomierski SL, Tseng LM *et al.* Preclinical testing of clinically applicable strategies for overcoming trastuzumab resistance caused by PTEN deficiency. *Clin Cancer Res* 2007; **13**: 5883–8.
17 Marinov M, Ziogas A, Pardo OE *et al.* AKT/mTOR pathway activation and BCL-2 family proteins modulate the sensitivity of human small cell lung cancer cells to RAD001. *Clin Cancer Res* 2009; **15**: 1277–87.
18 Motzer RJ, Escudier B, Oudard S *et al.* Efficacy of everolimus in advanced renal cell carcinoma: a double-blind, randomised, placebo-controlled phase III trial. *Lancet* 2008; **372**: 449–56.
19 Pavel ME, Hainsworth JD, Baudin E *et al.* Everolimus plus octreotide long-acting repeatable for the treatment of advanced neuroendocrine tumours associated with carcinoid syndrome (RADIANT-2): a randomised, placebo-controlled, phase 3 study. *Lancet* 2011; **378**: 2005–12.
20 Krueger DA, Care MM, Holland K *et al.* Everolimus for subependymal giant-cell astrocytomas in tuberous sclerosis. *N Engl J Med* 2010; **363**: 1801 –11.
21 Baselga J, Semiglazov V, van Dam P *et al.* Phase II randomized study of neoadjuvant everolimus plus letrozole compared with placebo plus letrozole

22 Jerusalem G, Fasolo A, Dieras V *et al.* Phase I trial of oral mTOR inhibitor everolimus in combination with trastuzumab and vinorelbine in pre-treated patients with HER2-overexpressing metastatic breast cancer. *Breast Cancer Res Treat* 2011; **125**: 447–55.
23 Perren A, Weng LP, Boag AH *et al.* Immunohistochemical evidence of loss of PTEN expression in primary ductal adenocarcinomas of the breast. *Am J Pathol* 1999; **155**: 1253–60.
24 Depowski PL, Rosenthal SI, Ross JS. Loss of expression of the PTEN gene protein product is associated with poor outcome in breast cancer. *Mod Pathol* 2001; **14**: 672–6.
25 Saal LH, Holm K, Maurer M *et al.* PIK3CA mutations correlate with hormone receptors, node metastasis, and ERBB2, and are mutually exclusive with PTEN loss in human breast carcinoma. *Cancer Res* 2005; **65**: 2554–9.
26 Koizumi F, Shimoyama T, Taguchi F, Saijo N, Nishio K. Establishment of a human non-small cell lung cancer cell line resistant to gefitinib. *Int J Cancer* 2005; **116**: 36–44.
27 Rosell R, Moran T, Queralt C *et al.* Screening for epidermal growth factor receptor mutations in lung cancer. *N Engl J Med* 2009; **361**: 958–67.
28 Samuels Y, Velculescu VE. Oncogenic mutations of PIK3CA in human cancers. *Cell Cycle* 2004; **3**: 1221–4.
29 Samuels Y, Wang Z, Bardelli A *et al.* High frequency of mutations of the PIK3CA gene in human cancers. *Science* 2004; **304**: 554.
30 Carpten JD, Faber AL, Horn C *et al.* A transforming mutation in the pleckstrin homology domain of AKT1 in cancer. *Nature* 2007; **448**: 439–44.
31 Saal LH, Gruvberger-Saal SK, Persson C *et al.* Recurrent gross mutations of the PTEN tumor suppressor gene in breast cancers with deficient DSB repair. *Nat Genet* 2008; **40**: 102–7.
32 Nielsen TO, Hsu FD, Jensen K *et al.* Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma. *Clin Cancer Res* 2004; **10**: 5367–74.
33 Mabuchi S, Kawase C, Altomare DA *et al.* mTOR is a promising therapeutic target both in cisplatin-sensitive and cisplatin-resistant clear cell carcinoma of the ovary. *Clin Cancer Res* 2009; **15**: 5404–13.
34 Di Nicolantonio F, Arena S, Tabernero J *et al.* Deregulation of the PI3K and KRAS signaling pathways in human cancer cells determines their response to everolimus. *J Clin Invest* 2010; **120**: 2858–66.
35 Voduc KD, Cheang MC, Tyldesley S, Gelmon K, Nielsen TO, Kennecke H. Breast cancer subtypes and the risk of local and regional relapse. *J Clin Oncol* 2010; **28**: 1684–91.
36 Neshat MS, Mellinghoff IK, Tran C *et al.* Enhanced sensitivity of PTEN-deficient tumors to inhibition of FRAP/mTOR. *Proc Natl Acad Sci USA* 2001; **98**: 10314–9.
37 Shi Y, Gera J, Hu L *et al.* Enhanced sensitivity of multiple myeloma cells containing PTEN mutations to CCI-779. *Cancer Res* 2002; **62**: 5027–34.
38 Galanis E, Buckner JC, Maurer MJ *et al.* Phase II trial of temsirolimus (CCI-779) in recurrent glioblastoma multiforme: a North Central Cancer Treatment Group Study. *J Clin Oncol* 2005; **23**: 5294–304.
39 Gori S, Sidoni A, Colozza M *et al.* EGFR, pMAPK, pAkt and PTEN status by immunohistochemistry: correlation with clinical outcome in HER2-positive metastatic breast cancer patients treated with trastuzumab. *Ann Oncol* 2009; **20**: 648–54.
40 Korsching E, Jeffrey SS, Meinerz W, Decker T, Boecker W, Buerger H. Basal carcinoma of the breast revisited: an old entity with new interpretations. *J Clin Pathol* 2008; **61**: 553–60.
41 Ryden L, Jirstrom K, Haglund M, Stal O, Ferno M. Epidermal growth factor receptor and vascular endothelial growth factor receptor 2 are specific biomarkers in triple-negative breast cancer. Results from a controlled randomized trial with long-term follow-up. *Breast Cancer Res Treat* 2010; **120**: 491 –8.
42 Mani SA, Guo W, Liao MJ *et al.* The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* 2008; **133**: 704–15.
43 Polyak K, Weinberg RA. Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. *Nat Rev Cancer* 2009; **9**: 265–73.

in patients with estrogen receptor-positive breast cancer. *J Clin Oncol* 2009; **27**: 2630–7.

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Circulating endothelial cells and other angiogenesis factors in pancreatic carcinoma patients receiving gemcitabine chemotherapy

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Abstract

Background: Pancreatic carcinoma is a significant cause of cancer-related death in developed countries. As the level of circulating endothelial cells (CECs) is known to increase in response to various cancers, we investigated the predictive potential of CEC levels and the association of these levels with the expression of proangiogenic factors in pancreatic carcinoma patients.

Methods: Pancreatic carcinoma patients receiving gemcitabine chemotherapy were prospectively assigned to this study. CEC levels were measured using the CellTracks system, and the plasma levels of several angiogenesis factors were measured using multiplex immunoassay. Associations between clinical outcomes and the levels of these factors were evaluated.

Results: Baseline CEC levels were markedly higher in pancreatic carcinoma patients (n = 37) than in healthy volunteers (n = 53). Moreover, these high CEC levels were associated with decreased overall survival (median, 297 days versus 143 days, $P < 0.001$) and progression-free survival (median, 150 days versus 64 days, $P = 0.008$), as well as with high vascular endothelial growth factor, interleukin (IL)-8, and IL-10 expression in the pancreatic carcinoma patients.

Conclusions: Several chemokines and proangiogenic factors correlate with the release of CECs, and the number of CECs detected may be a useful prognostic marker in pancreatic carcinoma patients undergoing gemcitabine chemotherapy.

Trial registration: UMIN000002323

Keywords: Pancreatic carcinoma, Circulating endothelial cells, Angiogenesis factors

Background

Pancreatic carcinoma is one of the most lethal tumors and is the fourth leading cause of cancer-related death in developed nations [1]. As pancreatic carcinoma has a high propensity for both local invasion and distant metastasis, surgery is precluded as a treatment for most patients who present with advanced-stage disease. These patients have a median survival of only 6 months and an overall 5-year survival of less than 5%. The prognosis for advanced pancreatic carcinoma patients is therefore extremely poor, and the impact of standard therapy is only modest, despite many advances that have improved the outcome of this disease.

Pancreatic carcinoma is not a grossly vascular tumor; however, it overexpresses multiple mitogenic growth factors that are also angiogenic, such as epidermal growth factor (EGF), hepatocyte growth factor (HGF), fibroblast growth factor (FGF), platelet-derived growth factor B chain (PDGF-BB), and vascular endothelial growth factor (VEGF). Angiogenesis often occurs in response to an imbalance in which proangiogenic factors predominate over antiangiogenic factors. For instance, VEGF expression has been shown to promote tumor growth in pancreatic carcinomas [2]. High VEGF expression is also

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associated with increased microvessel density [3] and is a predictor of poor outcomes and early tumor recurrence after curative resection [4]. Although agents that target the VEGF signaling pathway have been shown to inhibit tumor growth, metastasis, and angiogenesis [5], treating advanced pancreatic carcinoma patients with axitinib—a selective inhibitor of VEGF receptors 1, 2, and 3—in combination with gemcitabine was not found to improve overall survival in a phase 3 trial [6]. Despite this finding, proangiogenic factors remain an important therapeutic target for the treatment of pancreatic carcinoma.

Circulating endothelial cells (CECs) are mature cells that are not associated with vessel walls but are detached from the endothelium and circulate within peripheral blood. The number of CECs present in the blood has been found to increase in response to cardiovascular disease, vasculitis, infectious disease, and various cancers [7,8]. Indeed, the level of CECs has been recognized as a useful biomarker for vascular damage. It has also been reported that the number of CECs found in non-small cell lung cancer patients treated with carboplatin plus paclitaxel is a promising predictive marker of the clinical efficacy of these drugs [9]. We believe that CEC levels may also be a potential biomarker for pancreatic carcinoma; therefore, we investigated the levels of CECs found in patients with different severities of pancreatic carcinoma, as well as the effects of gemcitabine treatment on CEC levels. Furthermore, the associations between CEC levels and the expression levels of several factors involved in angiogenesis and neovascularization were also examined in this study.

Methods
Study approval
This prospective study was approved by the Institutional Review Board of the National Cancer Center, and written informed consent was obtained from all patients. This study is registered with the University Hospital Medical Information Network in Japan (UMIN; number UMIN000002323) and has been completed.

Patients and blood sample collection
A total of 37 chemotherapy-naïve patients with histologically or cytologically confirmed invasive ductal pancreatic carcinoma were prospectively enrolled in this study between April 2009 and March 2010 and received gemcitabine chemotherapy. Patients with coexisting infections and/or cardiovascular illness were excluded. The detailed history of all the patients was obtained and a physical examination was performed before beginning gemcitabine treatment. Pretreatment baseline laboratory parameters were also assessed for all patients. The baseline tumor status of each patient was evaluated using computed tomography (CT) scans of the chest, abdomen, and pelvis, while peripheral blood sampling was performed both prior to treatment initiation (baseline) and at day 28 ± 7 after starting chemotherapy. A dose of 1000 mg/m^2 gemcitabine was administered intravenously for 30 min on days 1, 8, and 15 of a 28-day cycle until disease progression, unacceptable toxicity, or patient refusal occurred. The data collected included those pertaining to standard demographics and disease characteristics, the date of initial treatment, the best response to treatment, date of progression, and the date of death or last follow-up. The tumors were evaluated every 6–8 weeks after starting each course of gemcitabine, and best responses were documented according to the Response Evaluation Criteria in Solid Tumors (RECIST).

CEC enumeration
Blood samples from advanced pancreatic carcinoma patients were drawn into 10 mL CellSave Preservative Tubes (Immunicon Corp. Huntingdon Valley, PA) for CEC enumeration. Samples were obtained both before starting chemotherapy (baseline) and at 28 ± 7 days after starting chemotherapy. Samples were kept at room temperature and processed within 42 h of collection. All of the evaluations were performed without knowledge of the clinical status of the patients. The CellTracks system (Veridex, LLC), which consists of the CellTracks AutoPrep system and the CellSpotter Analyzer system, was used for endothelial cell enumeration. In this system, CECs are defined as CD146$^+$/DAPI$^+$/CD105-PE$^+$/CD45APC$^-$ cells. Briefly, CD146$^+$ cells were captured immunomagnetically by using ferrofluids coated with CD146 antibodies. The enriched cells were then labeled with the nuclear dye 4 V, 6-diamidino-2-phenylindole (DAPI), CD105 antibodies were conjugated to phycoerythrin (CD105-PE), and the pan-leukocyte antibody CD45 was conjugated to allophycocyanin (CD45-APC). Cells with the DAPI$^+$/CD105$^+$/CD45$^-$ phenotype were enumerated. We evaluated morphological cell viability and excluded dead cells from the cell count. The number of CECs in each sample was determined twice, and the mean value was calculated.

Antibody suspension bead array system
Peripheral blood was drawn into prechilled tubes containing ethylenediaminetetraacetic acid; was immediately subjected to centrifugation at 1000 g and 4°C for 15 min, plasma was transferred to microtubes and subjected to further centrifugation at 10,000 g and 4°C for 10 min to remove contaminating platelets. Plasma samples were collected from patients before gemcitabine treatment was initiated and were stored at −80°C until they were used for testing. The plasma concentrations of 7 biological markers (interleukin [IL]-6, IL-8, IL-10,

PDGF-BB, VEGF, HGF, and SDF-1 alpha) were assayed in a subgroup of patients and control individuals by using the Bio-Plex suspension array system (Bio-Rad, Hercules, CA), which allows the simultaneous identification of cytokines in a 96-well filter plate. In brief, the appropriate cytokine standards and diluted plasma samples were added to a 96-well filter plate and incubated at room temperature for 30 min with antibodies chemically attached to fluorescent-labeled micro beads. After 3 filter washes, premixed detection antibodies were added to each well and incubated for 30 min. After 3 more washes, premixed streptavidin-phycoerythrin was added to each well and incubated for 10 min, followed by 3 more washes. The beads were then resuspended in

125 μL of assay buffer and the reaction mixture was quantified using the Bio-Plex protein array reader. Data were automatically processed and analyzed with Bio-Plex Manager Software 4.1 by using the standard curve obtained using a recombinant cytokine standard.

Statistical analyses

The Mann–Whitney test was used to compare the distributions of clinical factors and marker concentrations between patients with progressive disease (PD) and those without PD, stages III and IV disease, or recurrence. The survival time (progression-free survival [PFS] and overall survival [OS]) and clinical factors (age, gender, and Eastern Cooperative Oncology Group [ECOG] performance status

Table 1 Patient characteristics and CEC detection

		Mean CEC level 166 cells/4 mL	Range (2–1195 cells/4 mL)	Total	P^a
		≥ 166 cells/4 mL	<166 cells/4 mL		
		CEC^high	CEC^low		
		12	25	37	
Age	Over 70	8	10	18 (49%)	0.17
	Below 70	4	15	19 (51%)	
Sex	Male	7	17	24 (65%)	0.72
	Female	5	8	13 (35%)	
Stage	III	3	11	14 (38%)	0.59
	IV	8	12	20 (54%)	
	Recurrence	1	2	3 (8%)	
ECOG PS	0	5	18	23 (62%)	0.09
	1	6	4	10 (27%)	
	2	1	3	4 (11%)	
Pancreatic tumor location	Head	5	12	17 (46%)	>0.9
	Body	5	9	14 (38%)	
	Tail	2	4	6 (16%)	
CA19-9 (U/mL)	≥10,000	3	5	8 (22%)	>0.9
	< 10,000	9	20	29 (78%)	
CRP (mg/dL)	≥1.0	7	3	10 (27%)	<0.01
	<1.0	5	22	27 (73%)	
Histology	Poorly differentiated	5	9	14 (38%)	0.62
	Moderately differentiated	4	10	14 (38%)	
	Adenosquamous	1	0	1 (2%)	
	N.E (cytology only)	2	6	8 (22%)	
Tumor response	Partial response	2	2	4 (11%)	<0.05
	Stable disease	4	18	22 (59%)	
	Progressive disease	6	5	11 (30%)	
Second line therapy	S-1	6	12	18 (49%)	1
	Oxaliplatin + S-1	0	2	2 (5%)	
	No	6	11	17 (46%)	

^aP values were calculated for each variable using Fisher's exact test.
Abbreviations: CEC = circulating endothelial cell; ECOG = Eastern Cooperative Oncology Group; CA19-9 = carbohydrate antigen 19–9; CRP = C-reactive protein.
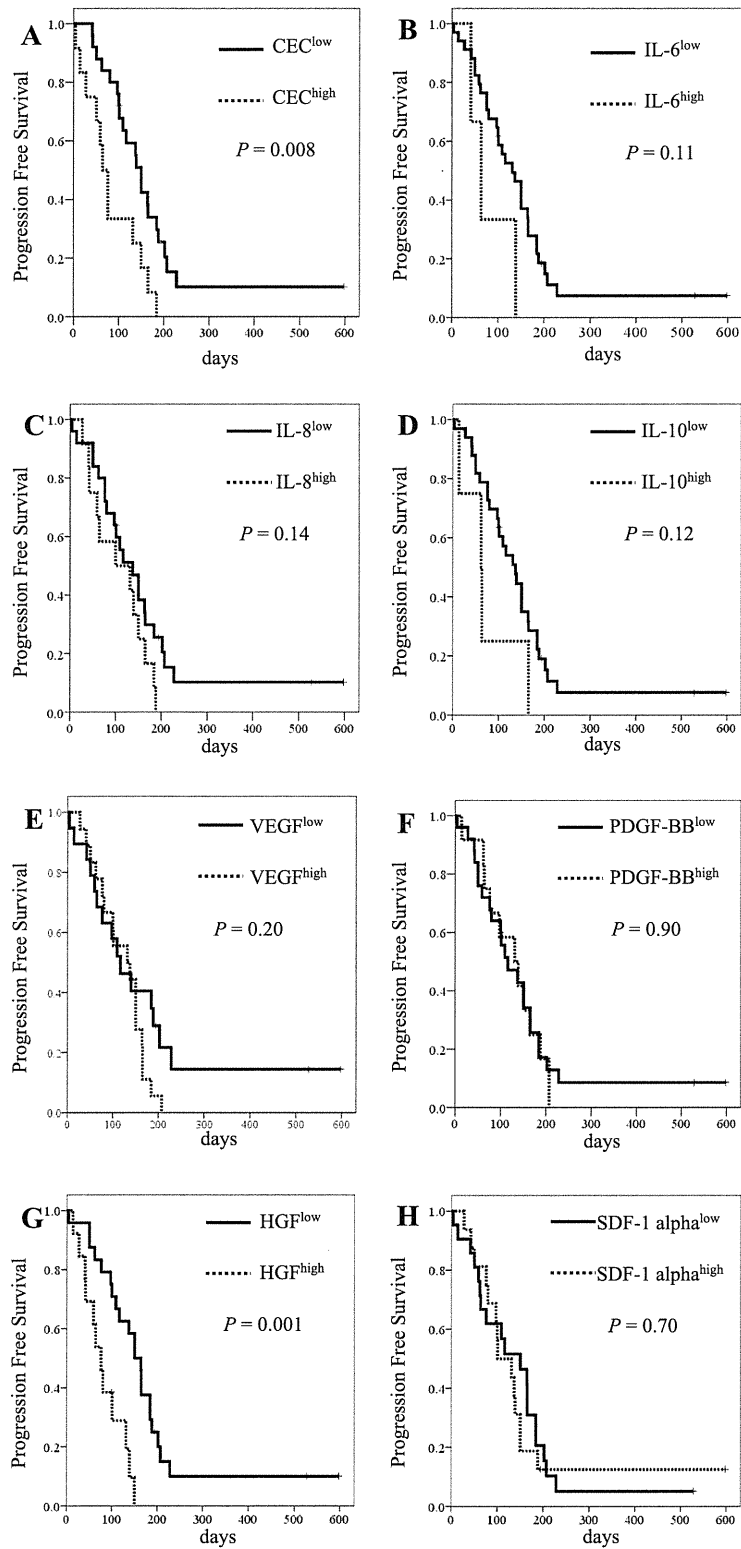
Figure 1 Kaplan-Meier curves for (A) progression-free survival with CEC counts, (B) progression-free survival with IL-6 levels, (C) progression-free survival with IL-8 levels, (D) progression-free survival with IL-10 levels, (E) progression-free survival with VEGF levels, (F) progression-free survival with PDGF-BB levels, (G) progression-free survival with HGF levels, and (H) progression-free survival with SDF-1 alpha levels. The cut-off points for the angiogenic factors were determined to be equal to or greater than these mean levels.

[PS], and clinical stage of the patients) were examined using the Cox proportional hazards model. The survival curves for PFS and OS were estimated using the Kaplan-Meier method. Kaplan-Meier curves were used only to determine the trends of the associations between the molecules and PFS/OS, as any determination of the optimal cutoff point for the molecules relative to PFS/OS was beyond the scope of the present study. All statistical analyses were performed using IBM SPSS Statistics 18 (IBM Corporation, Somers, NY, USA).

Results

Patient characteristics

A total of 37 patients with pancreatic carcinoma were prospectively enrolled in this study. Fourteen of these patients (38%) presented with locally advanced pancreatic carcinoma, 20 patients (54%) presented with metastases, and 3 patients (8%) were enrolled following recurrence after surgery. Twenty-three patients (62%) had ECOG PS0, 10 patients (27%) had ECOG PS1, and 4 patients (11%) had ECOG PS2. Histologically, 14 patients (38%) had poorly differentiated adenocarcinoma, 14 patients (38%) had moderately differentiated adenocarcinoma, 1 patient (2%) had an adenosquamous tumor, and 8 patients (22%) had cytological adenocarcinoma. No patient experienced a complete response to treatment. Four patients (11%) exhibited a partial response (PR) rate to treatment (11%), stable disease (SD) was observed in 22 patients (59%), and PD was observed in 11 patients (30%). Second-line therapy was administered to 20 patients (54%), whereby 18 patients (49%) received S-1 monotherapy and 2 patients (5%) received oxaliplatin and S-1 combination therapy (Table 1).

Baseline levels of CECs and angiogenic factors

The mean CEC level found in the pancreatic carcinoma patients was 166 cells/4 mL (range: 2–1195 cells/4 mL) while the median CEC level was 66 cells/4 mL. These CEC levels were higher than those of randomly-selected healthy volunteers ($P < 0.01$), as previously reported (n = 53, mean ± SD = 46.2 ± 86.3 cells/4 mL) [9]. In this study, the cut-off point of CEC^{high} was determined to be equal to or greater than 166 cells/4 mL while that of CEC^{low} was lower than 166 cells/4 mL. CEC^{high} was significantly associated with high levels of C-reactive protein (CRP) (over 1.0 mg/dL; $P < 0.01$). The median PFS was 64 days (95% confidence interval [CI], 45–83) in the CEC^{high} group, while that in the CEC^{low} group was 150 days (95% CI, 130–170; log-rank test; $P = 0.008$; Figure 1A). The median OS was 143 days (95% CI, 53–233) in the CEC^{high} group and 297 days (95% CI, 240–354) in the CEC^{low} group (log-rank test; $P < 0.001$; Figure 2A). Univariate analysis of CEC levels and clinical factors for OS was performed using the Cox

proportional hazard model. The hazard ratio (HR) for CEC levels (CEC^{high} versus CEC^{low}) was 5.18 (95% CI, 2.23–12.03; $P < 0.001$).

The mean levels of IL-6, IL-8, IL-10, PDGF-BB, VEGF, HGF, and SDF-1 alpha were found to be 19.3 pg/mL, 11.3 pg/mL, 7.82 pg/mL, 1127.5 pg/mL, 44.1 pg/mL, 471.3 pg/mL, and 110.6 pg/mL, respectively. The cut-off points for the angiogenic factors were determined to be equal to or greater than these mean levels, and the median PFS in HGF^{low} was longer than the HGF^{high} group ($P = 0.001$; Figure 1 G). However, other factors were not found to have statistical significance with regard to PFS. The median OS was longer in the case of IL-10 (112 days [95% CI, 50–173] in IL-10^{high} vs. 264 days [95% CI, 204–324] IL-10^{low}, log-rank test: $P = 0.003$; Figure 2d) and HGF (150 days [95% CI, 65–234] in HGF^{high} vs. 291 days [95% CI, 223–359] in HGF^{low}, log-rank test: $P = 0.01$; Figure 2 G).

Among the clinical factors that were examined in this study, a poor PS (PS 1 and 2), advanced stage (stage IV and recurrence), and high levels of IL-10, HGF, and CRP were significantly correlated with poor OS in univariate cox analysis, with HRs of 2.72 (95% CI, 1.29–5.70; $P = 0.008$), 2.21 (95% CI, 1.03–4.71; $P = 0.04$), 5.05 (95% CI, 1.55–16.39; $P = 0.007$), 2.52 (95% CI, 1.22–5.21; $P = 0.01$), and 2.49 (95% CI, 1.14–5.42; $P = 0.02$), respectively. In a multivariate Cox analysis model that included clinical stage, PS, CRP levels, CEC levels, IL-10 levels, and HGF levels, the number of CECs detected remained statistically stable at 0.05. The resulting HRs were 2.04 (95% CI, 0.78–5.35; $P = 0.15$), 2.58 (95% CI, 0.98–6.76; $P > 0.05$), 2.04 (95% CI, 0.62–6.76; $P = 0.24$), 5.14 (95% CI, 1.83–14.45, $P = 0.002$), 5.26 (95% CI, 1.26–22.22; $P = 0.02$) and 1.34 (95% CI, 0.46–3.91; $P = 0.59$), respectively (Table 2).

Changes in CEC number during treatment

The number of CECs was analyzed in 22 of the 37 patients at 28 ± 7 days after the start of gemcitabine therapy. The mean number of CECs detected in these patients after 28 ± 7 days was 133 cells/4 mL (range: 15–664 cells/4 mL), while the median number of CECs was 68 cells/4 mL. The absolute counts of CECs did not change significantly between day 1 and day 28 ± 7 of treatment (Mann–Whitney test, $P = 0.11$). Furthermore, a change in CEC counts from baseline to after 28 ± 7 days of treatment was not statistically associated with tumor response (Mann–Whitney test, $P > 0.05$, Figure 3).

Association between CEC number and blood angiogenic factors

The numbers of CECs were compared between non-PD (PR and SD, n = 26) and PD patients (n = 11) for
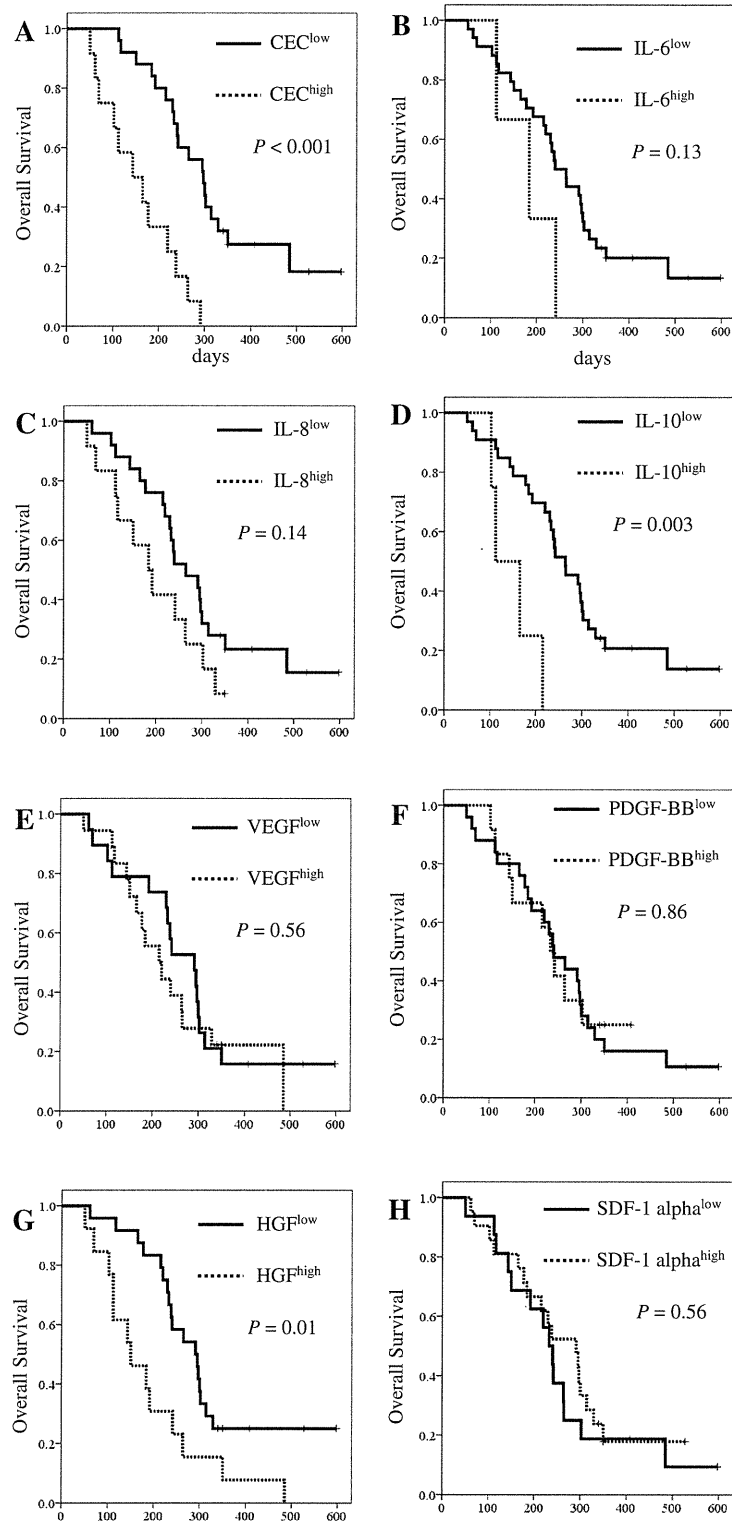
Figure 2 Kaplan-Meier curves for (A) overall survival with CEC counts, (B) overall survival with IL-6 levels, (C) overall survival with IL-8 levels, (D) overall survival with IL-10 levels, (E) overall survival with VEGF levels, (F) overall survival with PDGF-BB levels, (G) overall survival with HGF levels, and (H) overall survival with SDF-1 alpha levels. The cut-off points for the angiogenic factors were determined to be equal to or greater than these mean levels.

all markers. The baseline levels of CEC ($P = 0.03$), IL-6 ($P < 0.01$), and IL-10 ($P = 0.03$) were found to be significantly higher among patients with PD than among those with PR or SD. The blood concentrations of HGF ($P < 0.001$), IL-6 ($P < 0.01$), and IL-8 ($P < 0.001$) were also significantly higher among patients with clinical stage IV disease and recurrence than among those with stage III disease. When the association between CEC number and the expression of other angiogenic factors was examined, the number of CECs was found to correlate positively with the levels of VEGF ($r = 0.34$, $P = 0.04$), HGF ($r = 0.37$, $P = 0.02$), IL-8 ($r = 0.38$, $P = 0.02$), and IL-10 ($r = 0.45$, $P = 0.006$), suggesting that the number of CECs is related to the expression of these markers (Table 3).
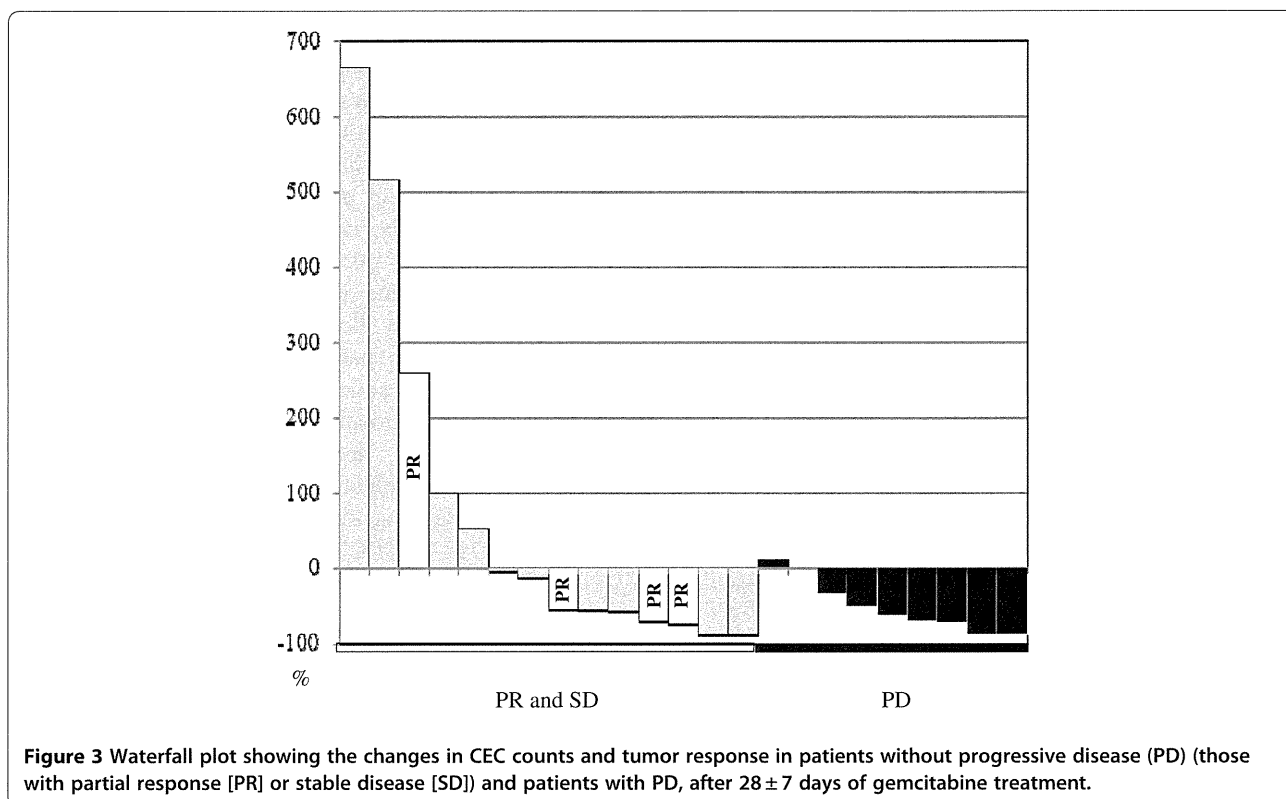
Discussions

In most cases, CECs are apoptotic or necrotic cells that are released into circulation as a byproduct of vascular turnover. In some cancer patients, the level of CECs is significantly higher than that of healthy individuals, and this increased level has been identified as a surrogate

marker of angiogenesis and anti-angiogenic drug activity [10,11]. The present study has shown that baseline CEC levels are markedly higher among pancreatic carcinoma patients than in healthy individuals. Our results also support the hypothesis that CEC levels are associated with clinical outcome in pancreatic carcinoma patients undergoing gemcitabine chemotherapy, and may be a prognostic factor for this disease. A previous study found that the baseline level of CECs, identified as $CD45^-CD31^+CD34^+$ by flow cytometry, was inversely associated with OS in patients who had gemcitabine-refractory metastatic pancreatic carcinoma and were treated with bevacizumab plus erlotinib [12]. CEC ($CD45^-CD31^+CD146^+$) detection by flow cytometry requires careful discrimination between blood cell populations with overlapping phenotypes showing hallmarks of T cells ($CD45^-CD31^-CD146^+$) and platelets ($CD45^- CD31^{high}CD146^-$). These cells populations show distinct regulation during cancer therapy, and their concomitant analysis may offer extended prognostic and predictive information [13].

Table 2 Univariate and multivariate Cox analyses of prognosis

Univariate analysis	HR	95% CI	*P*
Age: Over 70 vs. Below 70	0.52	0.25–1.13	0.1
Sex: Male vs. Female	1.00	0.48–2.08	0.99
Stage: IV + Recurrence vs. III	2.21	1.03–4.71	0.04
ECOG PS: 2 + 1 vs. 0	2.72	1.29–5.70	0.008
Pancreatic tumor location: Head vs. Others	0.94	0.46–1.90	0.86
CA19-9 (cut-off: 10,000 U/mL): CA19-9^{high} vs. CA19-9^{low}	1.77	0.75–4.15	0.19
CRP level (cut-off: 1.0 mg/dL): CRP^{high} vs. CRP^{low}	2.49	1.14–5.42	0.02
Histology: Poorly differentiated vs. Others	1.09	0.52–2.27	0.82
Second line therapy: Yes vs. No	0.61	0.30–1.24	0.17
CEC level (cut-off: 166 cells/4 mL): CEC^{high} vs. CEC^{low}	5.18	2.23–12.03	<0.001
IL-6 (cut-off: 19.3 pg/mL): IL-6^{high} vs. IL-6^{low}	2.52	0.73–8.64	0.14
IL-8 (cut-off: 11.3 pg/mL): IL-8^{high} vs. IL-8^{low}	1.74	0.82–3.67	0.15
IL-10 (cut-off: 7.82 pg/mL): IL-10^{high} vs. IL-10^{low}	5.05	1.55–16.39	0.007
VEGF (cut-off: 44.1 pg/mL): VEGF^{high} vs. VEGF^{low}	1.22	0.60–2.47	0.59
PDGF-BB (cut-off: 1127.5 pg/mL): PDGF-BB^{high} vs. PDGF-BB^{low}	0.93	0.43–2.04	0.86
HGF (cut-off: 471.3 pg/mL): HGF^{high} vs. HGF^{low}	2.52	1.22–5.21	0.01
SDF-1 alpha (cut-off: 110.6 pg/mL): SDF-1 alpha^{high} vs. SDF-1 alpha^{low}	1.23	0.60–2.53	0.56
Multivariate analysis	**HR**	**95% CI**	***P***
Stage: IV + Recurrence vs. III	2.04	0.78–5.35	0.15
ECOG PS: 2 + 1 vs. 0	2.58	0.98–6.76	>0.05
CRP level (cut-off: 1.0 mg/dL): CRP^{high} vs. CRP^{low}	2.04	0.62–6.76	0.24
CEC level (cut-off: 166 cells/4 mL): CEC^{high} vs. CEC^{low}	5.14	1.83–14.45	0.002
IL-10 (cut-off: 7.82 pg/mL): IL-10^{high} vs. IL-10^{low}	5.26	1.26–22.22	0.02
HGF (cut-off: 471.3 pg/mL): HGF^{high} vs. HGF^{low}	1.34	0.46–3.91	0.59

Abbreviations: HR = hazard ratio; CI = confidence interval; ECOG PS = Eastern Cooperative Oncology Group performance status; CEC = circulating endothelial cells; IL = interleukin; PDGF-BB = platelet-derived growth factor-B chain; VEGF = vascular endothelial growth factor; HGF = hepatocyte growth factor; CA19-9 = carbohydrate antigen 19–9; CRP = C-reactive protein; CEA = carcinoembryonic antigen.

Figure 3 Waterfall plot showing the changes in CEC counts and tumor response in patients without progressive disease (PD) (those with partial response [PR] or stable disease [SD]) and patients with PD, after 28 ± 7 days of gemcitabine treatment.

Our study also found the baseline level of CECs, as well as the levels of HGF, IL-6, and IL-10, which are associated with gemcitabine resistance or stemness, to be significantly higher among PD patients. Univariate Cox model analysis further demonstrated that PS, clinical stage, CRP levels, and CEC levels are all associated with the survival of pancreatic carcinoma patients, while multivariate Cox analysis showed that CEC and IL-10 levels are strongly associated with survival.

The number of CECs detectable in individuals has previously been found to be associated with the plasma levels of VCAM-1 and VEGF in cancer patients [14] [15]. Our findings further show that, in addition to VEGF, CEC levels are strongly associated with the expression levels of IL-8, IL-10, and HGF in pancreatic carcinoma patients. These molecules, among others, play important roles in tumor biology and have been implicated in several cellular phenotypes. Chemokines,

Table 3 Association between CECs and other factors

	Mean ± SD	Spearman's rank correlation coefficient	*P*
CEC (cells/4 mL)	166.2 ± 228.9	1	-
IL-6 (pg/mL)	19.3 ± 52.4	0.17	0.30
IL-8 (pg/mL)	11.3 ± 10.1	0.38	0.02
IL-10 (pg/mL)	7.82 ± 26.9	0.45	0.006
VEGF (pg/mL)	44.1 ± 38.8	0.34	0.04
PDGF-BB (pg/mL)	1,127.5 ± 941.5	0.24	0.16
HGF (pg/mL)	471.3 ± 249.0	0.37	0.02
SDF-1alpha (pg/mL)	110.6 ± 43.7	0.15	0.37
CRP (mg/dL)	1.9 ± 3.9	0.31	0.06
CA19-9 (U/mL)	18,229.1 ± 55,377.8	0.11	0.50
CEA (ng/mL)	18.3 ± 51.0	0.03	0.88

Abbreviations: CEC = Circulating endothelial cell; IL = interleukin; PDGF-BB = platelet-derived growth factor-B chain; VEGF = vascular endothelial growth factor; HGF = hepatocyte growth factor; CA19-9 = carbohydrate antigen 19-9; CRP = C-reactive protein; CEA = carcinoembryonic antigen.

including IL-8 and IL-10, are small peptides involved in controlling cell migration, particularly in leukocytes, during inflammation and the immune response. Chemokines are also important in tumor biology as they influence tumor growth, invasion, metastasis, and angiogenesis. For instance, VEGF, HGF and IL-8 significantly stimulate the proliferation, migration, and invasion of cancer cells. CEC are shed from vessels and this process may be amplified by an aberrant vascular turnover/remodeling associated with high local levels of VEGF required for CEC survival [16]. The chemokine SDF-1 has likewise been found to enhance the production of IL-8 by pancreatic cells in a paracrine manner [17]. Although our results did not indicate that SDF-1 levels were associated with CEC or IL-8 levels in the pancreatic cancer patients examined, it is likely that several of the proangiogenic factors examined in this study interact with each other to promote vascular turnover and remodeling, thereby leading to a higher number of CECs in the peripheral blood of cancer patients.

Drugs targeting angiogenesis, such as those that inhibit the VEGF pathway, have had a major impact in the treatment of many types of cancer. The VEGF pathway is also an independent prognostic factor for patient survival in pancreatic carcinoma. Although preclinical models have suggested that VEGF-VEGF receptor inhibitors would be effective in the treatment of pancreatic carcinoma, patients who received bevacizumab and axitinib therapy in addition to gemcitabine have not shown a survival advantage when compared to those treated with gemcitabine alone [6,18]. These results add to the increasing evidence that suggests that targeting VEGF signaling is an ineffective strategy in the treatment of pancreatic carcinoma. However, many antiangiogenic therapies modulate the expression levels of proangiogenic factors [19], and many factors are associated with tumor angiogenesis. Therefore, there are a variety of potential therapeutic targets that may be exploited in order to target angiogenesis, potentially including those examined in this study.

In advanced non-small cell lung cancer (NSCLC), patients with higher baseline CEC counts have PR/SD and longer PFS. It has also previously been reported that the elevated CEC numbers exhibited in NSCLC patients decrease following treatment with carboplatin in combination with paclitaxel [9]. Paclitaxel and docetaxel are categorized as mitotic spindle agents with potent antiangiogenic properties [20-22]. Therefore, it seems that the baseline CEC count is a promising predictor of clinical response to the carboplatin plus paclitaxel regimen, as well as of survival. However, although several other clinical studies that have examined CECs have also found chemotherapy to be associated with either an increase or decrease in CEC number [23,24], no association was detected between gemcitabine treatment and CEC

number in the pancreatic carcinoma patients in our study. Although gemcitabine has anti-angiogenic properties, higher baseline CEC levels were associated with PD in pancreatic carcinoma patients receiving gemcitabine therapy, and patients with high CEC counts exhibited poor clinical condition. It is therefore likely that the tumor type, anti-cancer drugs being administered, and the amount of time between the start of treatment and the time when CEC counts are obtained influence the number of CECs detected in cancer patients after treatment. In this study, we measured CEC levels before starting chemotherapy and at 28 ± 7 days after starting chemotherapy, the time of sampling might influence the changes of CEC level. Moreover, the diversity in literature regarding CEC up-or down-regulation during cancer therapy and the associated prognostic and predictive evidence might in part be explained by a differential focus on or by the lack of discrimination between these cell populations [13].

Conclusions

Although the number of patients examined in this study was small, and patients were recruited prospectively, this study, along with others, has shown the clinical importance of CEC number as a prognostic factor in advanced pancreatic carcinoma treated with gemcitabine chemotherapy, whereby high CEC counts are associated with poor prognosis. This study also found that elevated CEC counts are associated with the high expression levels of several chemokines and proangiogenic factors involved in the regulation of tumor immunological and angiogenic factors. Although this correlation between blood parameters is not proof of a causal relationship, these factors may provide viable therapeutic targets for the treatment of pancreatic carcinoma in the future. Further studies in a larger population will be required to confirm our findings.

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References
1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D: Global cancer statistics. *CA Cancer J Clin* 2011, **61**:69–90.
2. Korc M: Pathways for aberrant angiogenesis in pancreatic cancer. *Mol Cancer* 2003, **2**:8.
3. Seo Y, Baba H, Fukuda T, Takashima M, Sugimachi K: High expression of vascular endothelial growth factor is associated with liver metastasis and a poor prognosis for patients with ductal pancreatic adenocarcinoma. *Cancer* 2000, **88**(10):2239–2245.
4. Niedergethmann M, Hildenbrand R, Wostbrock B, Hartel M, Sturm JW, Richter A, Post S: High expression of vascular endothelial growth factor predicts early recurrence and poor prognosis after curative resection for ductal adenocarcinoma of the pancreas. *Pancreas* 2002, **25**(2):122–129.
5. Hicklin DJ, Ellis LM: Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. *J Clin Oncol* 2005, **23**(5):1011–1027.
6. Kindler HL, Ioka T, Richel DJ, Bennouna J, Letourneau R, Okusaka T, Funakoshi A, Furuse J, Park YS, Ohkawa S, et al: Axitinib plus gemcitabine versus placebo plus gemcitabine in patients with advanced pancreatic adenocarcinoma: a double-blind randomised phase 3 study. *Lancet Oncol* 2011, **12**(3):256–262.
7. Blann AD, Woywodt A, Bertolini F, Bull TM, Buyon JP, Clancy RM, Haubitz M, Hebbel RP, Lip GY, Mancuso P, et al: Circulating endothelial cells. *Biomarker of vascular disease. Thromb Haemost* 2005, **93**(2):228–235.
8. Goon PK, Lip GY, Boos CJ, Stonelake PS, Blann AD: Circulating endothelial cells, endothelial progenitor cells, and endothelial microparticles in cancer. *Neoplasia* 2006, **8**(2):79–88.
9. Kawaishi M, Fujiwara Y, Fukui T, Kato T, Yamada K, Ohe Y, Kunitoh H, Sekine I, Yamamoto N, Nokihara H, et al: Circulating endothelial cells in non-small cell lung cancer patients treated with carboplatin and paclitaxel. *J Thorac Oncol* 2009, **4**(2):208–213.
10. Farace F, Massard C, Borghi E, Bidart JM, Soria JC: Vascular disrupting therapy-induced mobilization of circulating endothelial progenitor cells. *Ann Oncol* 2007, **18**(8):1421–1422.
11. Bidard FC, Mathiot C, Delaloge S, Brain E, Giachetti S, de Cremoux P, Marty M, Pierga JY: Single circulating tumor cell detection and overall survival in nonmetastatic breast cancer. *Ann Oncol* 2010, **21**(4):729–733.
12. Ko AH, Venook AP, Bergsland EK, Kelley RK, Korn WM, Dito E, Schillinger B, Scott J, Hwang J, Tempero MA: A phase II study of bevacizumab plus erlotinib for gemcitabine-refractory metastatic pancreatic cancer. *Cancer Chemother Pharmacol* 2010, **66**(6):1051–1057.
13. Starlinger P, Brugger P, Reiter C, Schauer D, Sommerfeldt S, Tamandl D, Kuehrer I, Schoppmann SF, Gnant M, Brostjan C: Discrimination between circulating endothelial cells and blood cell populations with overlapping phenotype reveals distinct regulation and predictive potential in cancer therapy. *Neoplasia* 2011, **13**(10):980–990.
14. Mancuso P, Burlini A, Pruneri G, Goldhirsch A, Martinelli G, Bertolini F: Resting and activated endothelial cells are increased in the peripheral blood of cancer patients. *Blood* 2001, **97**(11):3658–3661.
15. Mancuso P, Calleri A, Cassi C, Gobbi A, Capillo M, Pruneri G, Martinelli G, Bertolini F: Circulating endothelial cells as a novel marker of angiogenesis. *Adv Exp Med Biol* 2003, **522**:83–97.
16. Tartour E, Pere H, Maillere B, Terme M, Merillon N, Taieb J, Sandoval F, Quintin-Colonna F, Lacerda K, Karadimou A, et al: Angiogenesis and immunity: a bidirectional link potentially relevant for the monitoring of antiangiogenic therapy and the development of novel therapeutic combination with immunotherapy. *Cancer Metastasis Rev* 2011, **30**(1):83–95.
17. Matsuo Y, Ochi N, Sawai H, Yasuda A, Takahashi H, Funahashi H, Takeyama H, Tong Z, Guha S: CXCL8/IL-8 and CXCL12/SDF-1alpha co-operatively promote invasiveness and angiogenesis in pancreatic cancer. *Int J Cancer* 2009, **124**(4):853–861.
18. Kindler HL, Niedzwiecki D, Hollis D, Sutherland S, Schrag D, Hurwitz H, Innocenti F, Mulcahy MF, O'Reilly E, Wozniak TF, et al: Gemcitabine plus bevacizumab compared with gemcitabine plus placebo in patients with advanced pancreatic cancer: phase III trial of the Cancer and Leukemia Group B (CALGB 80303). *J Clin Oncol* 2010, **28**(22):3617–3622.
19. Bertolini F, Marighetti P, Shaked Y: Cellular and soluble markers of tumor angiogenesis: from patient selection to the identification of the most appropriate postresistance therapy. *Biochim Biophys Acta* 2010, **1806**(2):131–137.
20. Belotti D, Vergani V, Drudis T, Borsotti P, Pitelli MR, Viale G, Giavazzi R, Taraboletti G: The microtubule-affecting drug paclitaxel has antiangiogenic activity. *Clin Cancer Res* 1996, **2**(11):1843–1849.
21. Hayot C, Farinelle S, De Decker R, Decaestecker C, Darro F, Kiss R, Van Damme M: In vitro pharmacological characterizations of the anti-angiogenic and anti-tumor cell migration properties mediated by microtubule-affecting drugs, with special emphasis on the organization of the actin cytoskeleton. *Int J Oncol* 2002, **21**(2):417–425.
22. Wang J, Lou P, Lesniewski R, Henkin J: Paclitaxel at ultra low concentrations inhibits angiogenesis without affecting cellular microtubule assembly. *Anticancer Drugs* 2003, **14**(1):13–19.
23. Beaudry P, Force J, Naumov GN, Wang A, Baker CH, Ryan A, Soker S, Johnson BE, Folkman J, Heymach JV: Differential effects of vascular endothelial growth factor receptor-2 inhibitor ZD6474 on circulating endothelial progenitors and mature circulating endothelial cells: implications for use as a surrogate marker of antiangiogenic activity. *Clin Cancer Res* 2005, **11**(9):3514–3522.
24. Hanrahan EO, Heymach JV: Vascular endothelial growth factor receptor tyrosine kinase inhibitors vandetanib (ZD6474) and AZD2171 in lung cancer. *Clin Cancer Res* 2007, **13**(15 Pt 2):s4617–s4622.