Inhibition of Placental Growth Factor in Renal Cell Carcinoma

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Abstract. *Background/Aim: Placental growth factor (PlGF) is up-regulated in major malignant diseases or following antiangiogenic therapy, although it is present in low levels under normal physiological conditions. TB403, a monoclonal antibody against PlGF, was investigated in clear cell renal cell carcinoma (ccRCC) xenografts since it has been proposed as a potential target in oncology. Materials and Methods: Human ccRCCs were implanted in athymic nude mice to evaluate the efficacy of TB403 and to excise xenograft tumors for molecular experiments. Results: TB403 did not significantly inhibit tumor growth in treatment-naïve or sunitinib-resistant ccRCC xenografts. Gene expression profiling resulted in over-expression of the C1orf38 gene, which induced immunoreactivity in macrophages. Angiogenesis PCR arrays showed that VEGFR-1 was not expressed in ccRCC xenografts. Conclusion: PlGF blockade did not have a broad antiangiogenic efficacy; however, it might be effective on-target in VEGFR1-expressing tumors. The inhibition of VEGF pathway may induce the activity of tumor-associated-macrophages for angiogenesis escape.*

Worldwide, 170,000 patients are newly-diagnosed with renal cell carcinoma (RCC) every year resulting in 72,000 deaths per year (1). The treatment of advanced clear cell RCC (ccRCC) has significantly changed over the past several years by inhibiting the vascular endothelial growth factor (VEGF) or mammalian target of rapamycin (mTOR) pathways (1-4). In particular, sunitinib is a first-line therapy for advanced ccRCC patients based on significant advantages over interferon-alpha (IFN-α) in a phase III trial (4). However, the development of ccRCC resistance to sunitinib is a major clinical problem. There is a need for the investigation of novel targets for antiangiogenic agents.

Placental growth factor (PlGF) is a VEGF homolog that exclusively binds Flt1 (VEGFR1) and co-receptor neuropilin-1 (NRP1), which does not contain a tyrosine kinase domain. PlGF, a disease-specific cytokine with low serum levels in healthy subjects, increases in malignant, inflammatory and ischemic disorders and is secreted by various cell types, including vascular endothelial cells, smooth muscle cells and many different tumor cells (5). It is a potential prognostic biomarker in breast, lung and colon cancers as higher PlGF circulating levels are associated with more aggressive disease (6-8). In colorectal cancer patients with metastatic disease, serum PlGF levels increase following the administration of bevacizumab in combination with chemotherapy (9). The fact that plasma PlGF concentrations is reported to increase in patients with RCC receiving sunitinib suggests a potential correlation between this mechanism and angiogenic rescue (10). PlGF may play a role in resistance to certain antiangiogenic therapies and its inhibition may overcome its resistance.

According to Fischer *et al*., treatment with an anti-PlGF monoclonal antibody (mAb) reduces microvascular density (MVD) and inhibits primary tumor growth in a variety of murine tumor models (11). However, in a contrasting study, blocking PlGF does not result in growth inhibition in most of their tumor models (12 murine and 3 human tumor cell lines), although the antibodies used were able to inhibit PlGF activity *in vivo* (12). Importantly, the anti-PlGF mAb (αPlGF) was shown to inhibit metastasis of B16F10

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Key Words: Placental growth factor (PlGF), antiangiogenic therapy, renal cell carcinoma, sunitinib resistance.

melanoma cells and the growth of a primary tumor murine cell line over-expressing VEGFR-1 (11, 13). In addition, efficacy in these models was not associated with a reduction in tumor MVD and, as a result, an alternative mechanism involving vascular normalization has been proposed.

TB403 (RO5323441) is a humanized recombinant immunoglobulin G isotype 1 (IgG1) mAb that binds to both human and murine PlGF and is not cross-reactive with human VEGF (hVEGF) (14). Several ongoing studies have been registered in the ClinicalTrial.gov database. For example, a study of TB403 monotherapy in patients with metastatic treatment, refractory colorectal or ovarian cancer (NCT01148758) and a phase I study evaluating TB403 in combination with bevacizumab in patients with recurrent glioblastoma (NCT01308684), are underway (8, 15).

This study aimed to investigate the therapeutic potential of TB403 in RCC xenograft models and sunitinib-resistant tumors and examine its underlying mechanism(s) through gene expression profile analysis before and after treatment.

Materials and Methods

Reagents. TB403 (Roche diagnostics, Penzberg, Germany) was prepared as a 25 mg/ml stock solution in histidine buffer (20 mmol/l histidine, 140 mmol/l sodium, pH6.0). Bevacizumab and sunitinib were purchased from the clinical pharmacy at the National Cancer Centre Singapore. All drugs were kept in aliquots and stored in –20˚C.

Cells and cell culture. 786-O, A-498 and Caki1 RCC cell lines were purchased from the American Type Culture Collection (ATTC, Manassas, VA, USA). SN12C cells were obtained from the National Cancer Institute (Bethesda, MD, USA). The cells were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (Gibco, Carlsbad, CA, USA) without antibiotics in a humidified incubator containing 5% CO_2 at 37˚C. All cell lines were used within 10 passages. The cells were passaged for less than six-months in our laboratory after receipt or resuscitation. No independent authentication of these cells was done by the authors.

Murine tumor xenografts. All experiments involving animals were conducted under approved protocols granted by the SingHealth Institutional Animal Care and Use Committee. Tumor subcutaneous implantation was performed as described previously (16). Six- to eight-week-old female athymic nude mice were obtained from the Animal Resource Centre (Canning Vale, Western Australia). All cell lines were inoculated with medium in the right flank of mice ($3\text{-}5\times10^6$ cells/mouse). Antibodies were administered by intraperitoneal injection once per week for four weeks at the doses indicated in the corresponding figure legends when tumors had reached an average volume of 200 to 300 mm^3. After four weeks, the experiment was terminated and tumors were resected for immunohistochemistry and microarray analysis. The procedure for the sunitinib resistant model was performed as described previously (17). Sunitinib was administered by oral gavage once daily at the dosages of 40 mg/kg (for 786-O xenografts) or 80 mg/kg (for SN12C xenografts) once tumors had grown to an average volume of 200 to 300 mm^3. The tumor growth ratio was determined by dividing the tumor volume measured at an indicated time by the tumor volume at the start of sunitinib treatment. Tumors that increased >25% of initial volume were considered sunitinib-resistant. Non–tumor-bearing mice were given a single administration of TB403, sunitinib, bevacizumab or anti-ragweed mAb at the doses indicated in the corresponding figure legends.

Immunohistochemistry. Tumors obtained from xenograft models were fixed in 10% neutral buffered formalin (10% formaldehyde, phosphate-buffered) overnight and embedded in paraffin. From each case, a representative tissue block consisting of predominantly viable tumor tissue was selected from hematoxylin and eosin staining. For immunohistochemical (IHC) studies, this validation was performed on the Leica Bond III automated system (Leica Microsystems, Wetzlar, Germany) as described previously (18). Specimens were deparaffinized and antigen was retrieved on the instrument. All slides were incubated with the first primary antibody, CD31 (SC-1506; Santa Cruz Biotech, TX, USA), at a dilution of 1:100 for 15 min, with post primary polymer for eight min, blocked with 3% hydrogen peroxide for five minutes and 3,3-diaminobenzidine (DAB, brown chromogen) for 10 minutes. The secondary antibody, (ab97049; ABCAM, Cambridge, UK), was incubated at a dilution of 1:500 for 15 minutes and hematoxylin-counterstained for five minutes. These incubations were performed at room temperature. Between incubations, sections were washed with Tris-buffered saline (bond wash solution). All scoring was carried out by a pathologist who was blinded to the other experimental outcome at the Histology Facility, Agency of Science, Technology and Research (A*STAR) in the assessment of immunohistochemical staining and tissue histology.

RNA extraction and microarray analysis. Total RNA was extracted from frozen xenograft tumor specimens using the TriPure Isolation Reagent (Roche Diagnostics, Penzberg, Germany) according to the manufacturer's instruction. After DNase treatment (Promega, Madison, WI, USA) to eliminate genomic DNA, RNA was further cleaned up using the Qiagen RNeasy Mini kit (Qiagen, Hilden, Germany). The quantity of the RNA was determined by an absorbance at 260nm with a Nanodrop (ThermoScientific, Waltham, MA, USA) and the integrity of the RNA was analysed by a Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) using the Agilent RNA 6000 Nano kit. Only samples with RNA integrity numbers (RINs) greater than 8.0 were used. cDNA was synthesized by the RT2 First Strand kit (SABiosciences, Hilden, Germany) following the company's instructions. Gene expression profiling was carried out using Human Affymetrix U133 plus 2.0 chips (Affymetrix Inc., Santa Clara, CA, USA). Data were robust multi-array average (RMA) normalized and analysed using the Partek Genomics Suite (Partek Inc., St. Louis, MO, USA) (19).

Angiogenesis PCR array. The Human Angiogenesis RT2 Profiler PCR Array and RT2 SyBR Green/ROX PCR Mastermix were purchased from SABiosciences and PCR was performed on a ABI7900HT, Fast Real-Time PCR system (Applied Biosystems) following the manufacturer's protocol. This platform is designed to profile the expression of 84 key genes related to angiogenesis including growth factors and receptors, chemokines, as well as cytokines. For PCR analysis, data were analysed using the ΔΔCt

method normalized to housekeeping genes (*e.g.*, *ACTB, B2M, GAPDH, HPRT* and *RPL13A*), where a significant threshold was defined as a 3-fold change in gene expression (20).

Human and murine ELISA. Human and murine PlGF, VEGF and sFlt1 were measured in cell culture supernatants and blood plasma using the Quantikine Immunoassay kit (R&D systems, Minneapolis, MN, USA) following the manufacturer's instructions.

Statistical analysis. Statistical analysis was performed using the GraphPad Prism, version 5.0 (GraphPad Software Inc., La Jolla, CA, USA). The ANOVA test was used for quantitative data that showed a normal distribution. Nonparametric data sets were analysed using the Kruskal-Wallis nonparametric test. *p*-Values ≤0.05 were considered significant.

Results

Anti-PlGF antibody does not inhibit growth of ccRCC xenografts. To investigate the effect of TB403 and the crosstalk between ccRCC tumor cells and their microenvironment, mice bearing ccRCC tumors were treated with TB403. Endothelial cells, fibroblasts and macrophages are implicated in the PlGF production. Thus, the use of a xenograft with specific ELISA allowed us to investigate PlGF expression derived from the tumor (human) or host (murine). There were no significant differences detected in ccRCC tumor growth between the control group and TB403-treated groups in any of the dosages tested (Figure 1A-1C). hPlGF was produced in low or non-detectable amounts in control treated tumors (<10 pg/ml, n=3), although circulating murine PlGF (mPlGF) levels were significantly elevated in the 30 mg/kg of the TB403 group compared to the control and the non-tumor bearing mice (healthy group) (Figure 1D). Plasma levels of human and murine VEGF were not affected following TB403 administration (Figure 1E-1F). To further evaluateTB403, an antibody against mPlGF, 5D11D4 was also investigated together with TB403 to compare their abilities to block ccRCC tumor growth (Figure 1G). We found that both TB403 and 5D11D4 failed to inhibit RCC tumor growth. Interestingly, the circulating mPlGF level significantly increased with TB403 administration (Figure 1H), whereas no increase in the 5D11D4 treatment group was detected. In addition, there was no body weight loss in the mice treated with TB403 or 5D11D4 (data not shown). These results indicate that TB403 does not inhibit ccRCC tumor growth and stimulates excess mPlGF production through host stromal cells.

Phenotypic resistance of ccRCC xenografts and elevated mPlGF level. Despite the efficient efficacy of sunitinib to ccRCC, the development of sunitinib resistance is a major clinical concern. We expected that PlGF would be a potential key regulator in sunitinib-resistant ccRCC. SN12C xenograft mice were treated with 80 mg/kg of sunitinib, as described in the Materials and Methods. Nineteen mice (out of 54) showed phenotypic resistance during the first round of this treatment and another 11 mice showed resistance during the second round treatment (Figure 2A). 786-O xenograft mice were treated with sunitinib at 40 mg/kg continuously for 35 days. Nineteen mice developed resistance on day 14 and another 10 mice also showed resistance by day 35 (Figure 2B). To assess if there were alterations in PlGF levels after the development of sunitinib-resistant tumors, ELISA-based analysis was conducted on murine plasma. We observed a significant increased mPlGF levels in the sunitinib-resistant group as compared with the sunitinib-sensitive group (Figure 2C). To identify if PlGF is functionally involved in the sunitinib resistance, resistant tumors were treated with TB403 monotherapy or combination therapy with sunitinib. Treatment with sunitinib-alone or sunitinib in combination with TB403 did not reduce the growth of resistant tumors any further in SN12C and 786-O xenografts; however, the tumor ratio was slightly increased in the TB403 treatment group ($p<0.05$) (Figure 2D-2E). Furthermore, the plasma levels of mPlGF were significantly higher in the TB403-treated group than the sunitinib-treated alone (Figure 2F-2G). These results indicate that TB403 had no effect on the growth of sunitinib-resistant ccRCC tumor xenografts and treatment with TB403 excessively increased plasma levels of mPlGF.

In addition, previous literature shows that in contrast to anti-VEGF mAb, αPlGF did not cause any significant reduction in tumor vasculature (12, 13). To determine if TB403 could affect angiogenesis in ccRCC tumors, MVD (CD31-positive vessels) was quantified in sections from A498 and 786-O tumors and SN12C sunitinib-resistant tumors at the endpoint of the treatment (Figure 3A-3C). Our findings showed, once more, that TB403 treatment did not cause a reduction in MVD.

Hierarchical cluster analysis of microarray and angiogenesis profiling arrays. To elucidate possible gene expression changes, especially angiogenesis related-genes in the tumor following TB403 administration, gene expression profiling was carried out using a human Affymetrix U133 plus 2.0 chip on TB403 or control ccRCC xenografts. The hierarchical clustering shows 63 genes with more than 1.3-fold change, although the expression levels of the *VEGF* family remained unchanged after TB403 administration (Figure 4). For example, two genes, *C1orf38* and *GLIPR1*, statistically have a significant difference in all three ccRCC models. Increased expression of C1orf38 is involved in macrophage inflammatory response and down-regulation of GLIPR1 is associated with cancer activity indicating that tumor-associated macrophage may be up-regulated to produce excess PlGF.

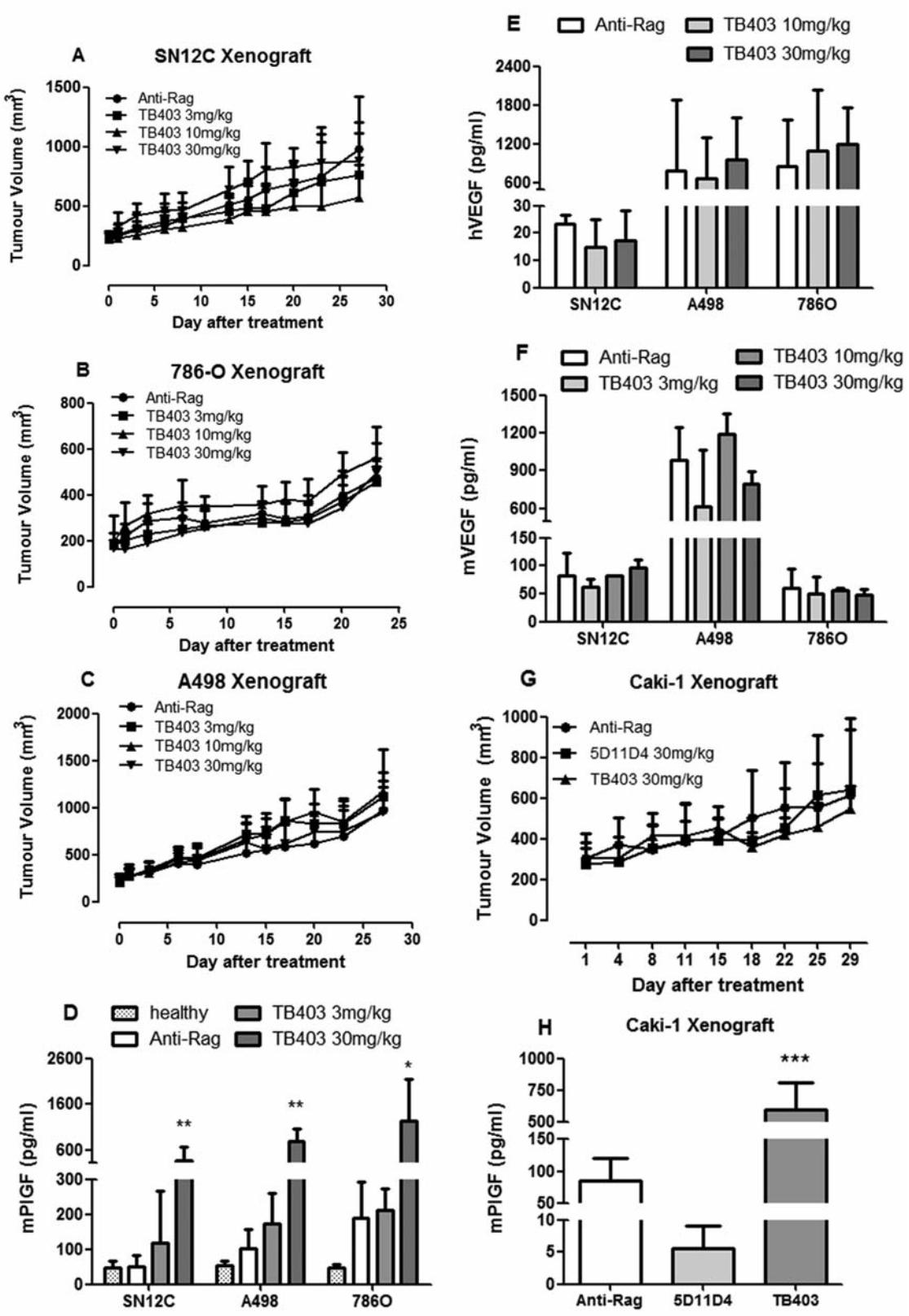

Figure 1. *TB403 treatment does not inhibit tumor growth. (A-C) Tumor size was measured and mice were randomly selected to one of four experimental groups (n=6-10). Error bars represent SD. (D-F) Plasma levels in mice bearing ccRCC tumors (n=5-9; *p<0.05, **p<0.01 versus control). (G-H) Effect of TB403, 5D11D4 or anti-ragweed on the growth of Caki1 tumor (n=4; ***p<0.001).*

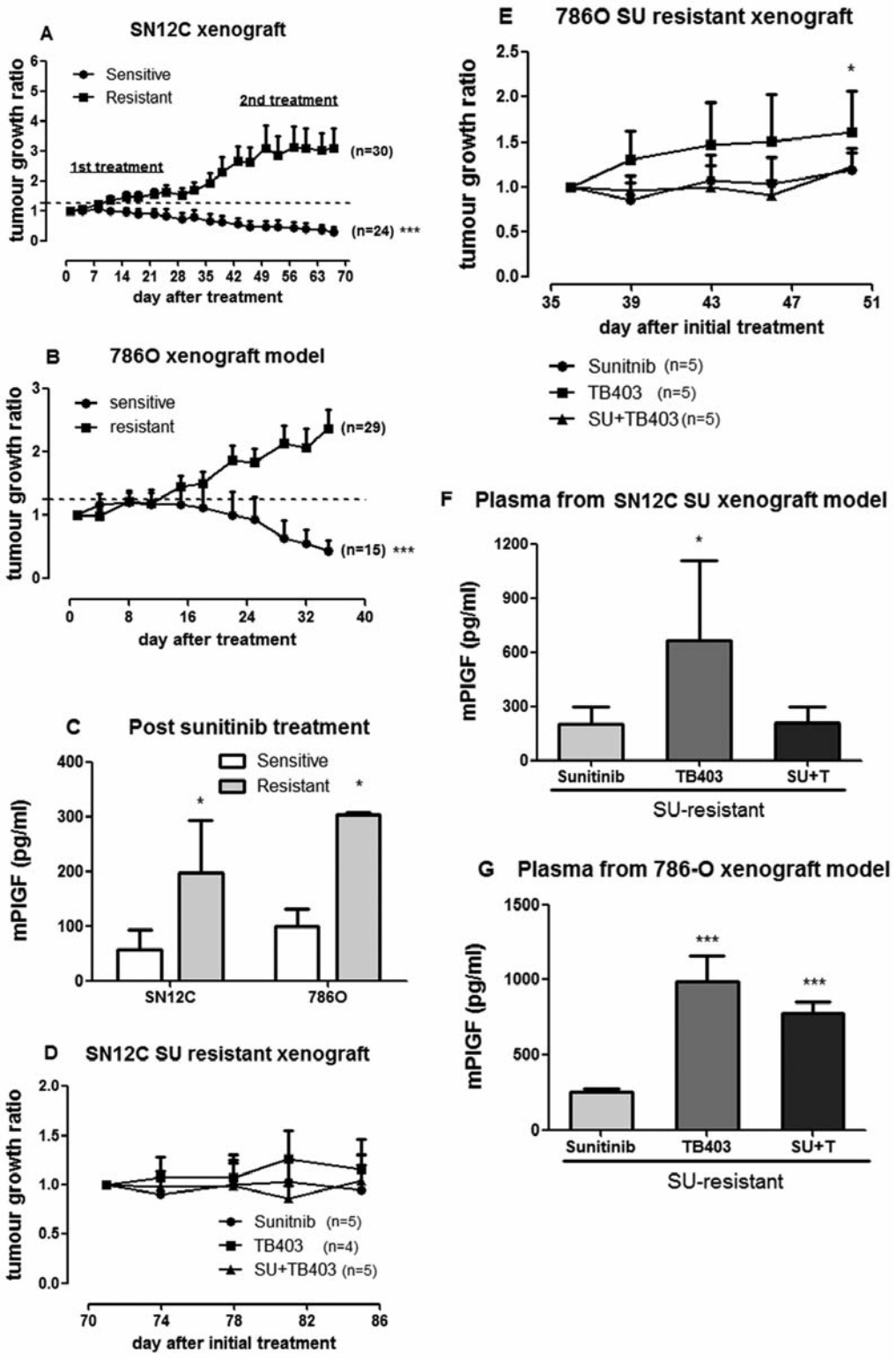

Figure 2. *The effect of TB403 for sunitinib-resistant RCC xenografts. (A) SN12C ccRCC xenografts were treated with sunitinib (80 mg/kg) intermittently. Tumor growth ratios for each group are presented as mean±95%CI (***p<0.001). (B) 786-O ccRCC xenograft were treated with sunitinib (40mg/kg) continuously for 35 days (***p<0.001). (C) Plasma mPlGF levels in these sunitinib-sensitive and -resistant mice (n=3-5). (D) All treatment for sunitinib-resistant SN12C xenograft mice started on day 71 and stopped on day 85. (E) Starting from day 36 to 50, the sunitinib-resistant 786-O xenograft mice were treated by three kinds of treatment. (F, G) Plasma mPlGF levels. Bars represent SD (*p<0.05, ***p<0.001 versus control).*

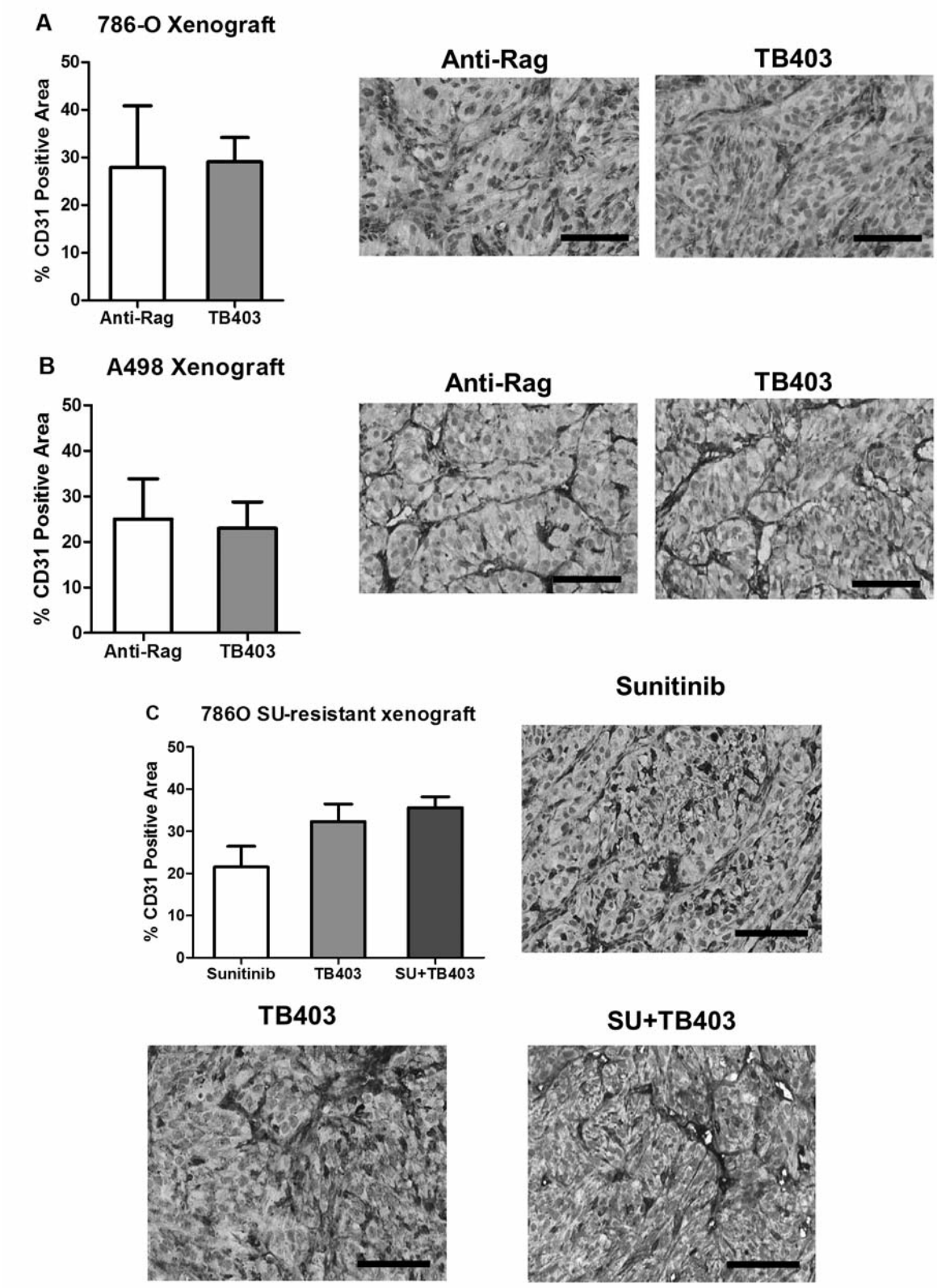

Figure 3. *TB403 does not induce antiangiogenesis. Tumor sections were analysed by immunofluorescence using CD31, an endothelial cell marker. Microvessel area in the tumor sections was measured and percent area was calculated in each group. Columns and mean represent bars and SD, respectively. (n=3-6) (total magnification for each image displayed, ×10). Scale bar, 0.10 mm.*

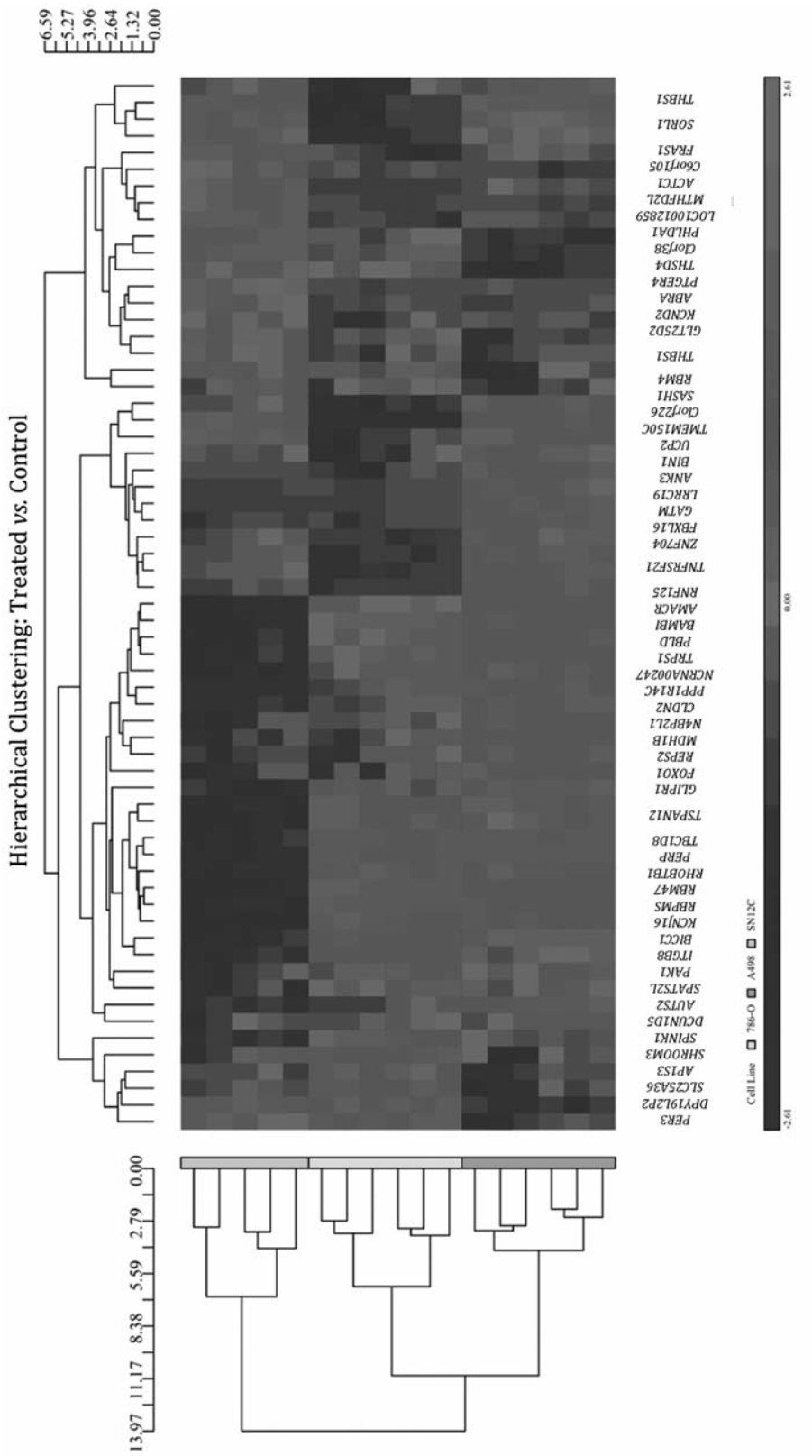

Figure 4. *Gene expression profile on RCC xenografts. Hierarchical clustering showing the identification of 63 genes (p<0.001) in SN12C, 786-O and A498 xenografts treated with TB403 or anti-ragweed mAb.*

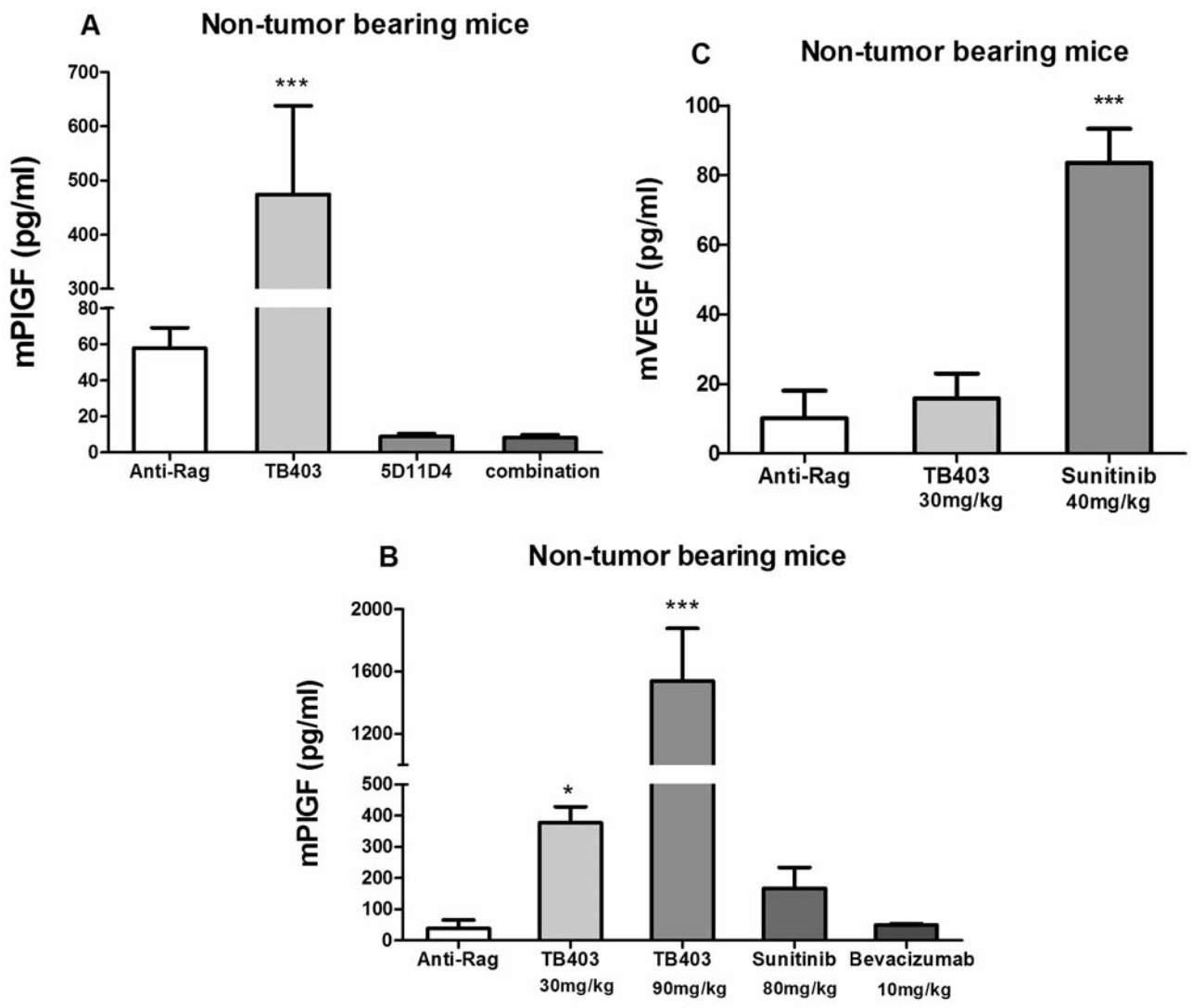

Figure 5. *Circulating mPlGF and mVEGF levels in non–tumor-bearing nude mice. (A) mPlGF levels in TB403 (30 mg/kg) group, control group (25 mg/kg anti-ragweed mAb), 5D11D4 (30 mg/kg) and combination (each 15 mg/kg of TB403 and 5D11D4). (B) Circulating mPlGF levels with different dose of TB403. (C) The levels of VEGF in plasma. (n=3-5; *p<0.05, ***p<0.001 versus control), error bars represent SD.*

For independent confirmation, RT^2 Profiler PCR array was performed to identify 84 different angiogenic mediators. Our data showed that the expression of VEGF family and other angiogenic factors, including angiopoietin family and epithelial growth factor, was not significantly changed in the TB403-treated tumors (Table I). As RCC tumors are known to be hypervascular tumors, VEGF-A was expressed in three RCC tumor models and was not affected by TB403. However, the mean cycle threshold (Ct) value of Flt1 was close to 35 cycles suggesting that Flt1 was not expressed in RCC tumors. Taken together, TB403 does not affect angiogenic gene expression in human ccRCC tumors and up-regulates genes related to macrophage and cancer activity.

mPlGF up-regulation is a host response to antiangiogenesis. To further investigate the role of host tissue in the secretion of mPlGF into the circulation, TB403 and 5D11D4 were administered to non–tumor bearing mice. The circulating mPlGF levels were significantly higher in the TB403 group than the control group. In contrast, circulating mPlGF was negligible in 5D11D4-treated and in the combination group (Figure 5A). Circulating mPlGF levels with TB403 also increased in a dose-dependent manner (Figure 5B). Consistent with previous literature, administration of sunitinib slightly increased PlGF levels; however, bevacizumab did not increase mPlGF levels in the circulation (11, 12). The circulating mVEGF levels were also evaluated in non–tumor-bearing

mice. The levels of VEGF did not change significantly after TB403 treatment (Figure 5C). These findings suggest that elevated mPlGF levels appear as a systemic response that occurred regardless of tumor implantation.

Discussion

This study provides the results of PlGF inhibition in ccRCC tumor models: First, the blockade of PlGF did not inhibit tumor growth of ccRCC xenografts. Second, PlGF expression was significantly up-regulated after antiangiogenesis therapy. Third, tumor-associated macrophages may have contributed to the increased PlGF level for angiogenesis and tumorigenesis escape. Forth, VEGFR1, Flt1 was not over-expressed in ccRCC cells.

The inhibition of internal biological pathways such as VEGF and mTOR has produced critical effects for metastatic ccRCC although tumors eventually acquire resistance to treatment due to microenvironmental change (3). Here we show that PlGF is produced only in negligible amounts by ccRCC. However, stromal cells produced increasing amounts of mPlGF after antiangiogenesis administration indicating that PlGF would be a key regulator for angiogenesis escape. As such, PlGF is a pleiotropic cytokine and drives a self-sustaining, reinforcing vicious cycle, whereby it would induce not only the recruitment and activation of macrophages and fibroblasts to release other angiogenic factors but also proliferation and migration of tumor cell expressing functional Flt1 (5). In contrast, it has been validated that PlGF is an antagonist to inhibit VEGF-A function through the formation of inactive VEGF-A/PlGF heterodimers, which reduce the amount of the active VEGF-A/VEGF-A homodimers (21, 22).

It has also been shown that anti-PlGF blockade reflects the modulation of innate immune responses rather than inhibition of angiogenesis and is likely due to direct inhibition of tumor growth that is dependent on VEGFR-1 or NRP1 signalling (23, 24). In our study, Flt1 was not over-expressed in RCC xenografts and PlGF reactivity was also not observed in the anti-VEGFR-resistant model; that is, TB403 was not efficacious in tumor growth, survival and intratumoral MVD in RCC xenografts. Importantly, plasma PlGF levels are increased in patients with colorectal cancer, RCC and glioblastoma by anti-VEGF therapies (9, 25). Consistent with our results, it is interesting that these therapies up-regulate VEGF-A and PlGF in mice without tumors and that production of PlGF by tumors has been implicated in the relapse of human xenograft tumors after radio-immunotherapy (26, 27). This suggests that PlGF contributes to the angiogenic escape induced by the blockade of the VEGF pathway.

Differences between the human and mouse species exist according to pharmacokinetic and pharmacodynamic parameters, immune responses and tumor microenvironment. It has been reported that a cross-reactive antibody to neutralize hPlGF and mPlGF inhibited ccRCC tumor growth; however, our results showed that both, TB403 and 5D11D4, failed to inhibit it (13). Since the reasons for such discrepancy remain unclear, it may be hypothesised that is dependent on the different specificity of the antibodies and species of mouse used (12-14). Furthermore, previous studies have reported that treatment with αPlGF at 50 mg/kg inhibits angiogenesis, tumor growth and metastasis of various tumors, although the dose used for other monoclonal antibodies (*i.e.*, bevacizumab) is usually within the range of 1-10 mg/kg in pre-clinical research and 5-15 mg/kg, clinically (11, 12). Thus, it is indicated that the effect of αPlGF might be due to off-target effects (11).

Higher serum mPlGF levels occurred in mice bearing tumor cells and in non–tumor-bearing mice following TB403 administration. It was also reported that the induction of angiogenic growth factors is a host response to antiangiogenic therapy following the administration of sunitinib or VEGF-A inhibitor in non–tumor-bearing mice and antiangiogenic therapy, which target both PlGF and VEGF, increases circulating mPlGF levels (12, 26). It was indicated that PlGF would be a potential regulator to induce another pathway for angiogenensis escape. Our findings suggested that TB403 might lead to the PlGF host response induced by anti-angiogenic therapies that will not be disallowed with the use of αPlGF. The increased secretion of PlGF into the circulation, upon administration of antiangiogenic therapies, could potentially promote more aggressive disease. PlGF over-expression conferred protection against apoptosis and induced a survival phenotype in brain tumor endothelial cells and macrophages in xenografts (5). The factors that may contribute to anti-angiogenic resistance and tumor escape are not clearly defined and have been the subject of several review articles (5, 28).

Table I. *Comparison of the respective Ct value.*

Ct Value	SN12C		A498		786-O	
Symbol	Anti-Rag	TB403	Anti-Rag	TB403	Anti-Rag	TB403
VEGFA	23.62	23.82	19.05	19.79	18.53	18.37
VEGFC	22.26	21.88	24.57	25.26	22.67	22.25
FIGF	30.06	30.45	28.46	29.20	30.97	30.58
KDR	29.17	28.89	34.92	33.81	36.17	35.62
PGF	34.04	35.06	30.86	31.31	25.29	24.91
FLT1	35.67	35.91	32.79	32.64	34.59	34.17
HIF1A	19.36	19.32	20.16	20.89	25.02	24.38
NRP1	23.04	22.99	21.25	21.44	21.94	22.22

The respective Ct values were obtained by qRT-PCR amplification for the expression of human angiogenesis in ccRCC xenograft tumors. Tumors were grown in immunodeficient mice and excised at the endpoint of this study. The experiment was repeated three times with three individual tumors in each of the cell lines.

In tumors, PlGF is not only produced by malignant cells but also by endothelial cells, pericytes, cancer-associated fibroblasts, tumor-associated macrophages and various other inflammatory cells in the tumor stroma (5, 11). Consistent with previous literature, our results indicated that PlGF levels would be induced by the recruitment of macrophages for tumor inflammation (29). The inhibition of the VEGF pathway probably up-regulates the expression of other chemoattractants, such as PlGF, FGF2 and G-CSF for macrophage activity (11, 30). In conclusion, PlGF blockade did not have a broad antiangiogenic or antitumor efficacy in RCC; however, it might be effective on-target in functional VEGFR1- or NRP1-expresseing tumors. The increased level of PlGF into the circulation, upon administration of antiangiogenic therapies, could be a potential biomarker. Similar therapeutic strategies may offer some clinical benefits in certain cancers. The approach of PlGF inhibition needs further investigation and should be preceded by molecular studies in the context of well-designed preclinical models for improved treatment of RCC in the future.

Financial Support

All funding: This work was supported by the Roche-SingHealth Translational and Research Hub grant entitled, "Role of placenta growth factor in antiangiogenic therapeutic related resistance in RCC".

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgements

The Authors would like to express their gratitude to Keith Rogers from the Histology facility for immunohistochemical staining, the Confocal and High Content Screening facility of Basement Shared Facilities (BSF), Agency of Science, Technology and Research (A*STAR) for their services. We would also like to thank Sabrina Noyes for proofreading and formatting the manuscript.

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Received September 5, 2014
Revised October 4, 2014
Accepted October 9, 2014