Acquired Drug Resistance to Vascular Endothelial Growth Factor Receptor 2 Tyrosine Kinase Inhibitor in Human Vascular Endothelial Cells

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Abstract. *Acquired resistance to antiangiogenic drugs has emerged as a potentially important issue in clinical settings; however, the underlying molecular and cellular mechanism of resistance to vascular endothelial growth factor receptor 2 (VEGFR2) tyrosine kinase inhibitor (TKI) remains largely unclear. We evaluated the cellular characteristics of human umbilical vein endothelial cell (HUVEC) clones, which are resistant to VEGFR2-TKI (Ki8751) to elucidate this mechanism of resistance to antiangiogenic drugs. Resistant HUVEC clones were 10-fold more resistant to VEGFR2-TKI than the parental cells and they exhibited an almost complete absence of VEGF-mediated cellular proliferation. The mRNA expression analysis revealed that expression of VEGFR1, VEGFR2 and VEGFR3 was lower in resistant clones, while that of several angiogenic ligands was increased. The protein expression of VEGFR2 was markedly down-regulated in two (R5 and R6 clone) out of five resistant clones. Focusing on the R5 clone, VEGF stimulation did not increase the phosphorylation of VEGFR2 or the dimerization of VEGFR2. The inhibition of phospho-AKT by VEGFR2-TKI was also weakened more than 10-fold in the R5 clone. Finally, a microarray analysis revealed that some angiogenesis-associated, and some angiogenesis-specific genes, including platelet endothelial cell adhesion molecule 1 (PECAM1)/CD31, homeobox A9 (HOXA9), and endothelial cell-specific molecule 1 (ESM1), were remarkably down-regulated in all the resistant clones compared with the parental cells. HUVEC clones resistant to VEGFR2-TKI exhibited down-regulation of VEGFR2, a decreased signal response to VEGF stimulation, and the loss of vascular endothelial markers. These results strongly suggest that an escape from VEGFR2 signaling-dependency is one of the cellular mechanisms of resistance to VEGFR2-TKI in vascular endothelial cells.*

Antiangiogenic inhibitors have exhibited a clinical benefit in cancer treatment; consequently, vascular endothelial growth factor (VEGF) and its receptor (VEGFR) are considered well-validated targets for cancer treatment (1). Bevacizumab, a VEGF-A-targeting monoclonal antibody, has demonstrated clinical benefit against metastatic colorectal cancer (CRC) and other solid types of cancer, while multikinase inhibitors targeting VEGFR2, including sunitinib and sorafenib, have shown clear positive results against renal cell carcinoma and hepatocellular carcinoma (2-4). Such evidence has accelerated the development of a wide variety of antiangiogenic inhibitors, and intensive clinical investigations are now underway.

Regarding sensitivity to antiangiogenic inhibitors, several promising predictive biomarkers of the clinical response have been identified (5). As a predictor of the response of metastatic breast cancer to bevacizumab treatment, an association between the *VEGF* genotype and the median overall survival (OS), as well as grade 3 or 4 hypertension, was shown (6). This previous study demonstrated that the *VEGF*-2578 AA genotype was associated with a superior median OS compared with that for the other genotypes combined. Meanwhile, the baseline plasma soluble VEGFR1, plasma VEGF, placental growth factor (PlGF), and interleukin 6 (IL-6) levels during treatment and the number of circulating endothelial cells (CECs) after treatment were significantly correlated with the clinical outcome (7). However, the mechanism of acquired resistance to antiangiogenic inhibitors, and biomarkers for treatment response remain largely unclear. One of the most popular hypotheses involves the use or activation of 'alternative proangiogenic pathways' that enable an escape from VEGF-VEGFR signaling

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(5, 8, 9). Generally, antiangiogenic drugs act on vascular endothelial cells, which are non-cancerous, non-mutated and genetically normal, while many kinase inhibitors target cancer cells that harbor active mutations that lead to oncogene addiction in the cells (10). Therefore, early drug resistance to antiangiogenic inhibitors in vascular endothelial cells was thought to be unlikely. However, recent clinical experiments have shown that the effect of antiangiogenic inhibitors does not persist as long as expected; in other words, drug resistance during an early phase of treatment has been observed in clinical settings (5). How and why vascular endothelial cells acquire drug resistance is now an important issue in antiangiogenic therapy, and an understanding of the underlying molecular mechanism may enable the identification of biomarkers for monitoring drug resistance or developing future improvements in the efficacy of drugs inhibiting alternative pathways.

In the present study, we investigated the mechanism of acquired resistant to Ki8751 hydrate, a potent VEGFR2-TKI, in human umbilical vein endothelial cells (HUVECs).

Materials and Methods

Reagents. Ki8751 hydrate, which is a potent inhibitor of VEGFR2 tyrosine kinase with an IC_{50} value for VEGFR2 of 0.9 nM (11), was purchased from Sigma Aldrich (St. Louis, MO, USA).

Cell cultures. HUVECs were purchased from Kurabo (Osaka, Japan) and maintained in Humedia (Kurabo) supplemented with 2% fetal bovine serum, 5 ng/ml of fibroblast growth factor 2 (FGF-B), 10 ng/ml of epidermal growth factor (EGF), 10 μg/ml of heparin, 1 μg/ml of hydrocortisone, 2 ng/ml of VEGF (R&D Systems, Minneapolis, MN, USA), and antibiotics. The cells were cultured in a humidified atmosphere of 5% CO_2 at 37˚C.

Immortalized HUVECs. We retrovirally introduced full-length human telomerase reverse transcriptase (hTERT) cDNA into HUVECs, as previously described (12, 13). The titer of the viral vector was calculated by counting the enhanced green fluorescent protein (EGFP)-positive cells that were infected by serial dilutions of virus-containing media, and the multiplicity of infection (MOI) was then determined. Stable virally transfected HUVECs were designated as immortalized HUVECs.

Ki8751-resistant clones in HUVECs. The methods used to establish clones resistant to a specific agent (gefitinib and 12-*O*-tetradecanoylphorbol 13-acetate, TPA) have been described previously (14, 15). Immortalized HUVECs were exposed to 2.5 μg/ml of *N*-methyl-*N*'-nitro-nitrosoguanidine (MNNG) for 24 h and then the medium was replaced with fresh medium containing a high dose of Ki8751 (5 μM). After exposure to Ki8751 for 4 days, the cells were washed and cultured in drug-free medium for 2 weeks. Colonies derived from a single cell were selected and cultured. A month later, the sensitivity of the resistant clones when exposed to Ki8751 was examined using a growth inhibition assay. The six resistant HUVEC clones obtained using the above-described method were designated as R1 to R6. The R3 clone was omitted from the analysis because its cellular growth gradually decreased and eventually stopped.

Immunoblotting. Western blot analyse were performed as previously described (16). The following antibodies were used: HER2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), HER3 antibody (Upstate Biotechnology, Lake Placid, NY), VEGFR2, platelet-derived growth factor receptor, beta (PDGFRβ), met proto-oncogene (hepatocyte growth factor receptor, MET), EGFR, p-VEGFR2, Akt, p-Akt, mitogen-activated protein kinase (MAPK), p-MAPK, phospho-protein kinase C (p-PKC), phospholipase C gamma (p-PLCγ), phospho-PLCγ1, p-p38MAPK, p38MAPK, β-actin antibody and HRP-conjugated secondary antibody (Cell Signaling Technology, Beverly, MA, USA). The sub-confluent cells were stimulated with VEGF at several concentrations for 10 min, and the cells were analyzed. The experiment was performed in duplicate.

Cell growth assay, chemical cross-linking assay and real-time reverse-transcription PCR. The methods were performed as previously described (16). The primers used are shown in Table I.

Microarray analysis. For the DNA microarray analysis, 0.5 μg of total RNA was amplified and labeled using an Amino Allyl MessageAmp™ II aRNA Amplification kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. Each sample of aRNA labeled with Cy3 and reference aRNA labeled with Cy5 were cohybridized with the 3D-Gene™ Human Oligo Chip 25k (Toray Industries, Inc., Tokyo, Japan) at 37˚C for 16 h (17). After hybridization, each DNA chip was washed and dried. The hybridization signals derived from Cy3 and Cy5 were scanned using Scan Array Express (PerkinElmer, Waltham, MA, USA). The scanned image was analyzed using GenePix® Pro (MDS Analytical Technologies, Sunnyvale, CA, USA). The microarray data was analyzed using BRB Array Tools software, Ver. 3.3.0 (http://linus.nci.nih.gov/BRB-ArrayTools.html), which was developed by Dr. Richard Simon and Dr. Amy Peng, as previously described (12, 16, 18).

Statistical analysis. The statistical analyses were performed using Microsoft Excel (Microsoft, Redmond, WA, USA) to calculate the standard deviation (SD) and to test for statistically significant differences between the samples using Student's *t*-test. A *p*-value <0.05 was considered statistically significant.

Results

Establishment of HUVEC clones resistant to a VEGFR2 tyrosine kinase inhibitor. Since HUVECs are derived from normal cells and their passage number is strictly limited to 5-8 times, obtaining clones that are resistant to anticancer drugs is difficult. We introduced the *TERT* gene into HUVECs to obtain immortalized HUVECs. The immortalized HUVECs were capable of undergoing an unlimited number of passages, and their cellular characteristics, such as the expression levels of VEGFRs and the VEGF-mediated cellular response, were the same as those of the original HUVECs (Figure 1). Clones resistant to VEGFR-TKI Ki8751 were established from the immortalized HUVECs using a previously described method (14, 15). The phenotype change observed in the resistant

Table I. *The primers used for the real-time RT-PCR.*

Genes	F-primer	R-primer
VEGFR1	CAGGGCAACAAGCCCGTTA	CCACATGGTGCGTCTCAAATTC
VEGFR2	CCAGGCAACGTAAGTGTTCGAG	GGGACCCACGTCCTAAACAAAG
VEGFR3	CTTGTCTGCTACAGCTTCCAGGTG	ATGTTCCGAGCAGCCAGGTC
PDGFRA	GTGCGAAGACTGAGCCAGATTG	CGATAAACAGAATGCTTGAGCTGTG
PDGFRB	GGACCTGCTATGAGGCTTTGGA	ACAAATGTGCAACCACCTGGAA
FGFR1	GAAGGCATCATTTGGTGAACAGAA	GGTAGGGCCAAGGCAGAAATTA
FGFR2	AATGCTGCTGTCAGACGATTGTTC	GTTGACGTAATGACAGGGTTGCAC
FGFR3	TTTGGACTTCAAAGCAAGCTGGTA	CTAATAACATCGGAACCTGCACACA
FGFR4	ACTGTGGCCGTCAAGATGCTC	ACACCAAGCAGGTTGATGATGTTC
VEGFA	TCACAGGTACAGGGATGAGGACAC	TCCTGGGCAACTCAGAAGCA
VEGFB	GCTTAGAGCTCAACCCAGACACC	CAAGTCACCCTGCTGAGTCTGAA
VEGFC	CAGCACGAGCTACCTCAGCAAG	TTTAGACATGCATCGGCAGGAA
PDGFA	AAGGCCTAGGGAGTCAGGTAAA	GTACATCCATGTCCCAGGAAAG
PDGFB	TAGCCTGCCTGATCCCTGAA	TCTGTGGTCTTAGCCATGGAGTC
HGF	GTAAATGGGATTCCAACACGAACAA	TGTCGTGCAGTAAGAACCCAACTC
ANGPT1	CTTCAACATCTGGAACATGTGATGG	TGGTTCTTGAACTGCATTCTGCTG
ANGPT2	GGACACACCACAAATGGCATCTAC	CCATCCTCACGTCGCTGAATAA
FGF1	GACCCAGGTGGCTAGCAAATTAGA	AGGCCTTTACATGGCATCAGTATCA
FGF2	GTGTGCTAACCGTTACCTGGCTATG	CCAGTTCGTTTCAGTGCCACA
FGF7	TGTGGTTGACCTATACGACCAGGA	CTCTGCCCTCAATATGCTCACAAG
CXCL1	GAACATCCAAAGTGTGAACGTGAAG	TTCAGGAACAGCCACCAGTGAG
CXCL2	CCACACTCAAGAATGGGCAGA	CCTTCAGGAACAGCCACCAATA
CXCL5	CAGTGCTCCAAGGTGGAAGTG	TGGGTTCAGAGACCTCCAGA
CXCL6	AAGTTTGTCTGGACCCGGAAG	AAACTGCTCCGCTGAAGACTG
CXCL7	CTTGCAGGTGCTGCTGCTTC	CATGCAGCGGAGTTCAGCATA
IL8	ACACTGCGCCAACACAGAAATTA	TTTGCTTGAAGTTTCACTGGCATC
PGF	CTCCTACGTGGAGCTGACGTTCT	GCAGTCTGTGGGTCTCTGCTTCT
FIGF	TGCCAGAAGCACAAGCTATTTCAC	TGCCACTTGCACATGGTCTG
PECAM1	GACGTGCAGTACACGGAAGTTCA	GTGCATCTGGCCTTGCTGTC
HOXA9	AGAATGAGAGCGGCGGAGAC	GGTCCCTGGTGAGGTACATGTTG
ESM1	TCCCGGCTGTGATTTCTGAG	ACCATGCATCACATTTGGTCTTC
GAPD	GCACCGTCAAGGCTGAGAAC	ATGGTGGTGAAGACGCCAGT

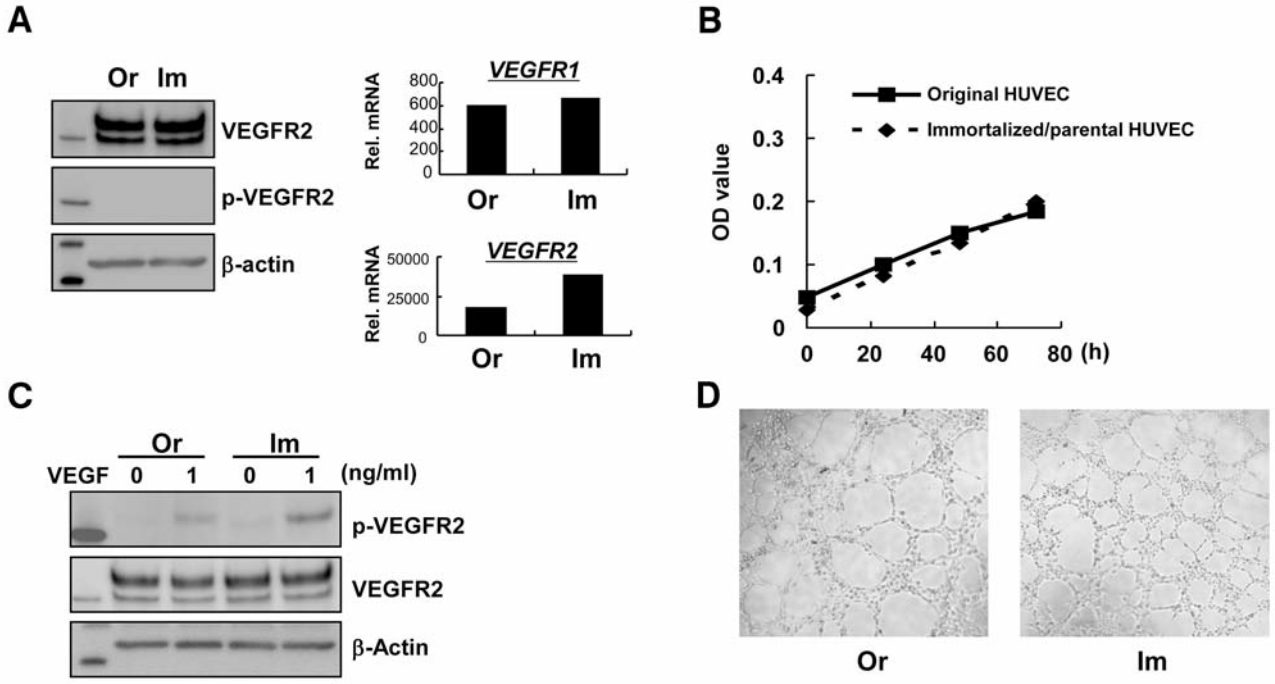
clones seemed to reflect a gradual restoration to the former parental HUVEC state over a period of ~5 months; thus, we considered the resistant clones to be semi-stable or reversible clones, presumably without genetic alterations.

A growth inhibition assay showed that the VEGFR-TKI-resistant clones were about 10-fold more resistant to Ki8751 than were the parental cells (Figure 2a). The cellular growth of the resistant clones did not respond to VEGF stimulation, whereas VEGF stimulation significantly increased the growth of the parental cells (Figure 2b). These results indicate that established resistant clones were resistant to Ki8751 and that they had lost their ability to undergo VEGF-mediated cellular growth, which is a key phenotypic characteristic of vascular endothelial cells.

Down-regulation of VEGFR2 expression in resistant clones. We examined the mRNA expression levels of several tyrosine kinase receptors such as *VEGFR1, VEGFR2, VEGFR3, PDGFRA, PDGFRB, FGFR1, FGFR2, FGFR3* and *FGFR4* (target receptors of multi-targeting VEGFR2-TKI) using real-time RT-PCR (Figure 3a). The expression of *VEGFR1, VEGFR2, VEGFR3* and *FGFR4* were markedly reduced in resistant clones. Because these baseline expression levels were much lower than that of *VEGFR2* and because of the pivotal biological roles of VEGFR2 in vascular endothelial cells, we speculated that the down-regulation of *VEGFR2* at the transcriptional level was one of the mechanisms responsible for resistance to VEGFR2-TKI.

On the other hand, the expression analysis of angiogenic ligands revealed that *ANGPT1, ANGPT2, CXCL5, CXCL6* and *CXCL7* were increased in resistant clones, whereas those of PGF (placental growth factor), *PDGFA* and *PDGFB* were decreased (Table II). The expression of *VEGFA, VEGFB, VEGFC* and *VEGFD* were unchanged in resistant clones.

Next, we examined the protein expression levels of several tyrosine kinase receptors such as VEGFR2 and PDGFRβ, as well as EGFR, HER2, HER3 and MET, known determinant receptors of resistance to EGFR-TKI, which are likely to be involved in sensitivity to VEGFR2-TKI. The expression levels of most of the receptors were unchanged in the



Figures 1. *Cellular characteristics of the immortalized HUVEC and the original HUVEC. A: Western blot analysis for the expression of VEGFR2, phospho-VEGF2 and β-actin in the original and immortalized/parental HUVECs. Real-time RT-PCR analysis for mRNA expression of VEGFR1, VEGFR2 in the original and immortalized/parental HUVECs. Or: Original HUVECs, Im: immortalized/parental HUVEC. Rel mRNA: mRNA expression levels normalized using GAPD. B: Growth curve of the original HUVECs (soild line) and immortalized/parental HUVECs (dotted line). Cells were seeded at a density of 2×10^3 cells/well in 96-well plates. Cell proliferation was estimated by measuring at 570 nm in 24 h intervals up to 72 h. C: VEGF stimulation to the original and immortalized/parental HUVECs. Cells were stimulated with the indicated concentrations of VEGF for 10 min. b-Actin was used as a control. D: Tube formation assay to the original and immortalized HUVEC. A total of 40 μl of Matrigel (BD Bioscience, San Jose, CA, USA) and 20 μl of PBS were mixed and incubated in 96-well plates. After the gel had solidified, a 100-μl volume of HUVECs cells (2×10^4 cells/well) was seeded onto the plates with 10 ng/ml of VEGF-A and the 96-well plates were incubated for 4 h. Capillary morphogenesis was evaluated under a microscope (Olympus, Tokyo, Japan).*

resistant clones compared with the levels in the parental cells. However, the expression of VEGFR2 protein was markedly down-regulated in two (R5 and R6 clones) out of five resistant clones (Figure 3b). The expressions were only slightly down-regulated in the other clones (R1, R2 and R4).

A phosphorylation and protein expression analysis showed that the phosphorylation level of AKT was slightly increased in resistant clones, except in the R1 clone, while that of MAPK was slightly increased in all the resistant clones (Figure 3c). These baseline differences in the phosphorylation status were likely specific to the resistant clones. The expression level of phospho-PKC was generally equivalent to that of the parental cells.

Decreased response to VEGF stimulation in resistant clones. To determine whether the downregulation of VEGFR2 in the resistant clones mediated the lower response to VEGF stimulation, we examined the response of the VEGFR2 signaling pathway to VEGF stimulation in dose and time course experiments. The phosphorylation of VEGFR2 (Tyr 1175, 1212 and 951) was dose-dependently increased in the parental cells after VEGF stimulation, while a slightly weakened response was observed in R2 and R4 clones (Figure 4a). Notably, VEGF stimulation had almost no effect on the phosphorylation level of VEGFR2 in the R5 clone. The phosphorylation levels of AKT and MAPK tended to be higher at baseline in the resistant clones, and a weakened response to VEGF stimulation was observed. Similar results were observed in the time-course analysis (Figure 4b).

Next, we focused on the R5 clone because of the marked down-regulation of VEGFR2 compared to the other clones. We evaluated the amount of VEGFR2 dimerization, which represents the strength of VEGFR2 signal activation. VEGFR2 dimerization was observed in parental cells, whereas dimerization was not observed in the R5 clone (Figure 4c). These results suggested that the resistant clone exhibited a decrease in the signal response to VEGF stimulation compared with the parental cells.

Inhibition of phospho-VEGFR2 and downstream molecules by Ki8751. We examined the differences in the Ki8751-induced inhibitory effects on the phosphorylation of

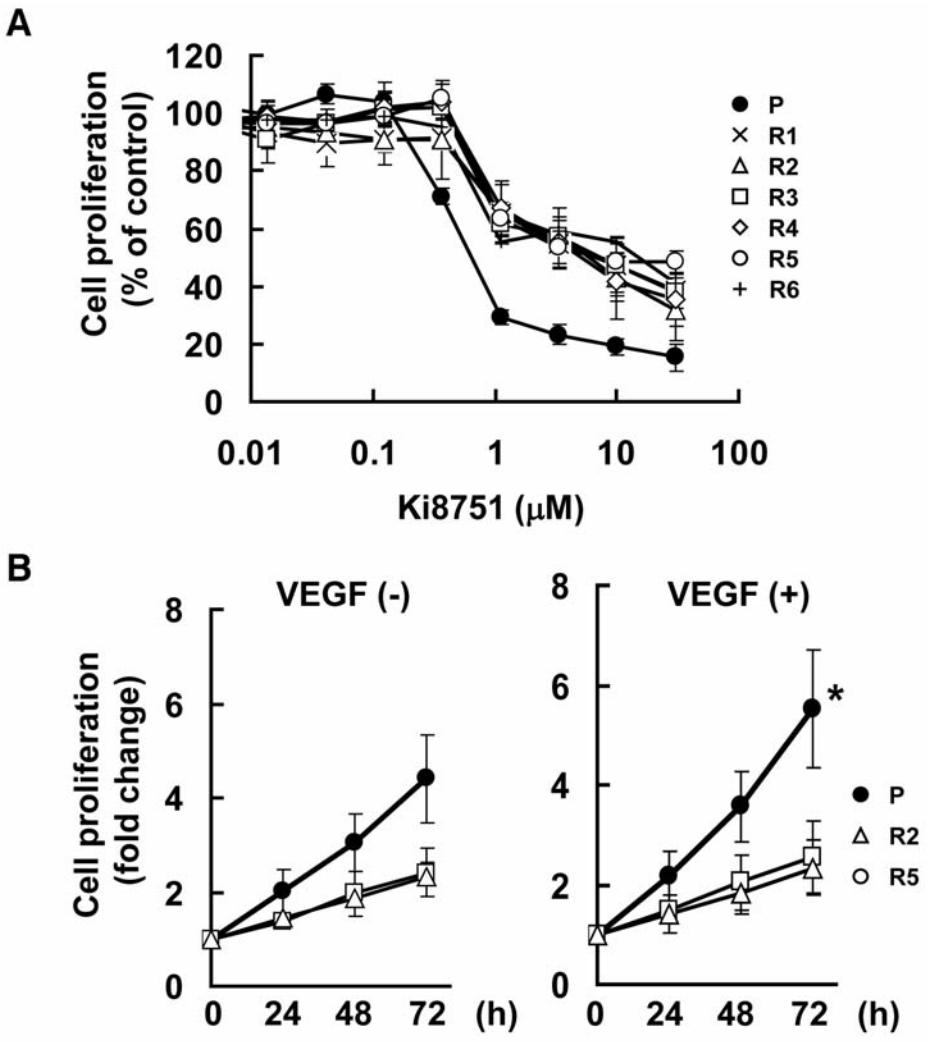


Figure 2. *Human umbilical vein endothelial cells (HUVECs) that had been immortalized through the introduction of the TERT gene were exposed to N-methyl-N'-nitro-nitrosoguanidine for 24 h and the medium was replaced with fresh medium containing a high dose of Ki8751 (5 μM) for 4 days. The cells were then washed and cultured in drug-free medium for 2 weeks. Colonies derived from a single cell were selected and cultured. The six resistant HUVEC clones obtained in this manner were designated as R1 to R6. The R3 clone was omitted from the analysis because its cellular growth gradually decreased and then stopped. A: Growth inhibitory effect of Ki8751 evaluated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. B: Growth curves of parental, R2 and R5 cells with, and without VEGF. The cells were seeded at a density of 2×10^3 cells/well in 96-well plates for 0, 24, 48 and 72 h in the presence, and absence of 10 ng/ml of VEGF. The MTT assay was conducted in triplicate. P: Parental HUVEC, R: resistant HUVEC clones. *VEGF (–) vs. (+), $p<0.05$.*

VEGFR2 and its downstream molecules in the parental cells and the R5 clone (Figure 5). Ki8751 (1 nM) completely inhibited the phosphorylation of VEGFR2 in the parental cells, while a basal level of VEGFR2 phosphorylation was not detected in the R5 clone. Meanwhile, the phosphorylation of AKT was inhibited by ~50% when parental cells were treated with 1 nM of Ki8751, whereas a similar percentage of AKT phosphorylation was achieved in the R5 clone when a dose of 100 nM was used, indicating that the inhibitory effect of Ki8751 against AKT phosphorylation was 10- to 100-fold different between parental and resistant cells. No differences in the inhibition of phospho-MAPK were observed, while basal levels of phospho-p38-MAPK and phospho-PLC-γ were low and with no response to VEGF stimulation in the R5 clone (Figure 5). Because the phosphorylation levels of p38-MAPK and PLC-γ are regulated by VEGFR2, inactivation of the VEGFR2 signaling pathway may mediate these results in the R5 clone. We found that the inhibitory effect of Ki8751 against phospho-AKT was decreased in the R5 clone.

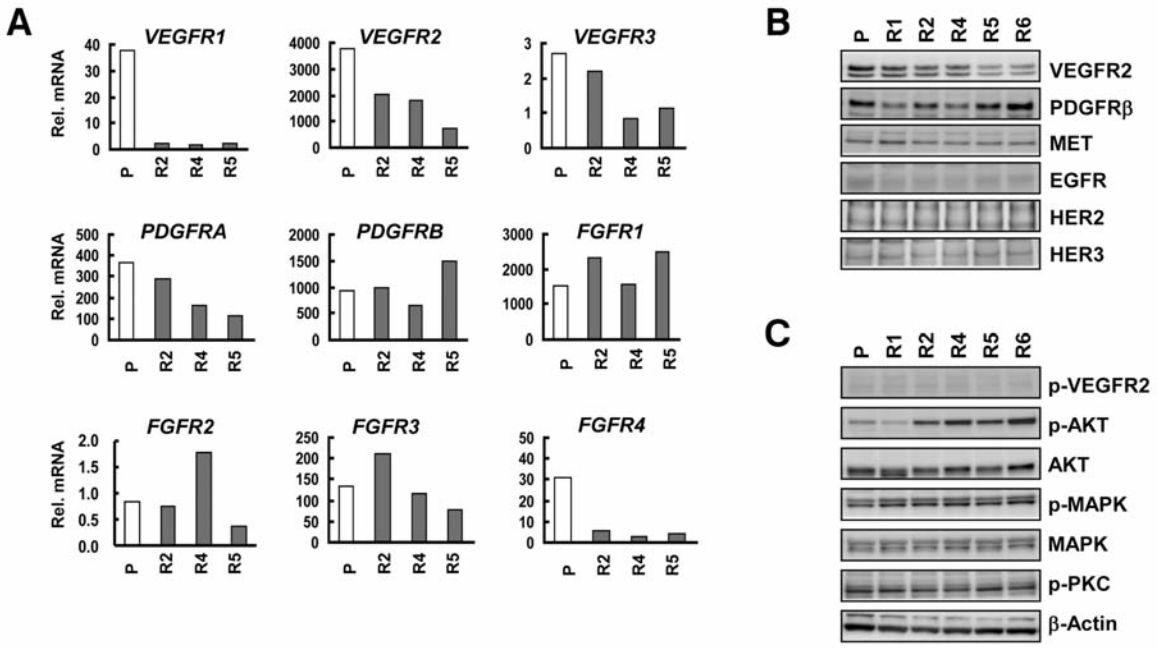


Figure 3. *VEGFR2 expression was down-regulated in the resistant clones. A: The mRNA expression levels of VEGFR1, VEGFR2, VEGFR3, PDGFRA, PDGFRB, FGFR1, FGFR2, FGFR3 and FGFR4 were determined using real-time RT-PCR. B: Western blot analysis for VEGFR2, PDGFR-β, MET, EGFR, HER2 and HER3 in the parental and resistant clones. C: Phosphorylation and protein expression levels of VEGFR2 and its downstream molecules. P: Parental HUVEC; R: resistant HUVEC clones; Rel mRNA: mRNA expression levels normalized using GAPD.*

Resistant clones lose vascular endothelial markers. A lower expression of VEGFR2 and a lower sensitivity to VEGFR2-TKI were observed in the resistant clones. One may speculate that the VEGFR2 signaling pathway, which plays a pivotal role in vascular endothelial properties, had become inessential for cell survival or cellular proliferation in the resistant clones. To detect expression changes in the resistant clones, we performed a microarray analysis of the parental cells and the R1, R2, R4 and R5 resistant clones. Forty-six genes were identified as being differentially expressed by more than 20-fold between the parental and resistant clones (Figure 6a). Thirty-nine genes were down-regulated in the resistant clones, and seven genes were up-regulated. Interestingly, the microarray analysis revealed *that platelet endothelial cell adhesion molecule precursor* (*PECAM1*), also known as *CD31*, was down-regulated in the resistant clones by 100-fold decrease compared with the parental cells. Other down-regulated genes included the chondroitin sulfate/dermatan sulfate proteoglycan *endothelial cell-specific molecule 1* (*ESM-1*, also known as endocan), and *homeobox A9* (*HOXA9*), which is involved in the formation of new vessels. The signal intensity of the array data is shown (Figure 6b). Real-time RT-PCR confirmed the down-regulation of the mRNA expression levels of *PECAM1, ESM1* and *HOXA9* (Figure 6c). The down-regulation of these vascular endothelial cell-specific genes suggests that resistant clones lose their vascular endothelial markers and presumably escape from VEGFR2 signaling-dependency.

In conclusion, an escape phenomenon that does not require the utilization of VEGF-VEGFR2 signaling and the loss of vascular endothelial-specific markers is at least partly involved in the mechanism of acquired drug resistance to VEGFR-TKI in vascular endothelial cells.

Discussion

The expression level of VEGFR2 and its phosphorylation was down-regulated in resistant HUVEC clones, and the degree of down-regulation seemed to be largely distinguished into two types: severe (R5 and R6) and mild (R1, R2 and R4) (Figures 3 and 4). These different expression levels might be associated with different mechanisms of resistance to VEGFR2-TKI in the two groups. We observed a decreased response to VEGF stimulation and the marked down-regulation of VEGFR2 expression in R5 and R6 clones. These results suggest that the down-regulation of VEGFR2 at the transcriptional level might be at least one of the mechanisms of drug resistance to VEGFR2-TKI, although the alternative signaling pathway remains undetermined.

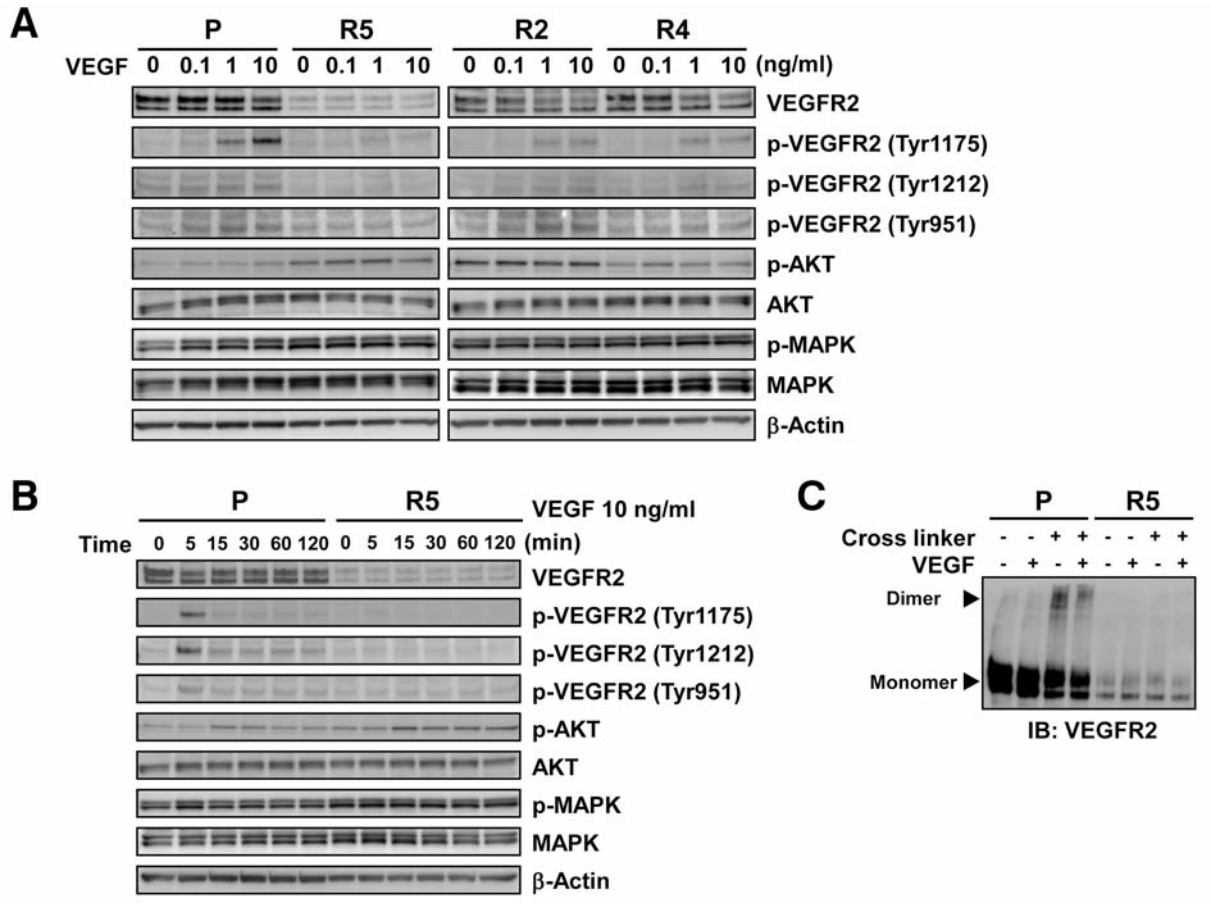


Figure 4. *The response to VEGF stimulation was lowered in the resistant HUVEC clones. A, B: The cells were stimulated with the indicated concentrations of VEGF for 10 min or with 10 ng/ml of VEGF at the indicated times. C: The total amount of VEGFR2 dimerization was evaluated in the parental cells and R5 clones using the cross-link method with, and without exposure to 10 ng/ml of VEGF. The arrowheads indicate dimerized or monomers of VEGFR2. P: Parental HUVEC, R: resistant HUVEC clones.*

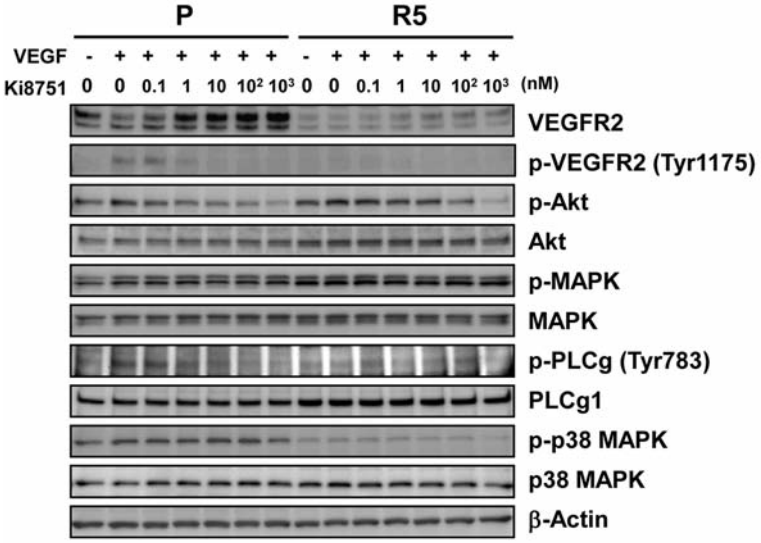


Figure 5. *Inhibition of the phosphorylation of VEGFR2 and its downstream molecules by Ki8751. Cells were exposed to the indicated concentrations of Ki8751 for 3 h and then stimulated with 10 ng/ml of VEGF for 10 min. The cells were then collected and used in a Western blot analysis. β-Actin was used as a control.*

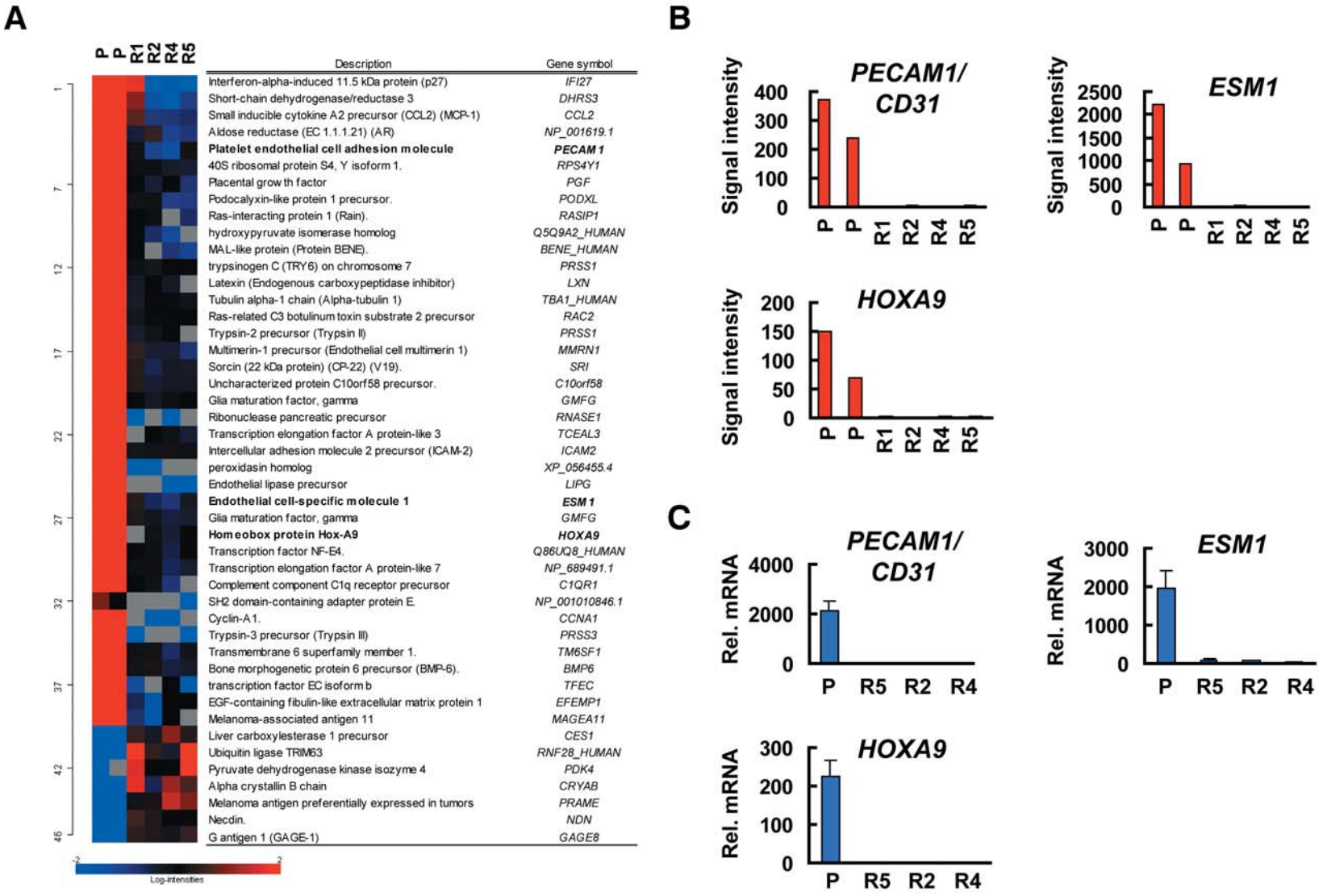


Figure 6. *The resistant clones lost the expression of endothelial cell-specific markers. A: A microarray analysis was performed for the parental and resistant clones. Genes that were differentially expressed between the parental and resistant clones by more than 20-fold are shown. B, C: The mRNA expression levels of PECAM1, ESM1 and HOXA9 are shown using the microarray and real-time RT-PCR data. Error bars represent the SDs of three independent experiments. Rel mRNA: mRNA expression levels normalized using GAPD.*

Table II. *The mRNA expression levels of the angiogenic ligands in parental and resistant HUVEC clones determined by real time RT-PCR.*

	P		R2	R4	R5
Gene	Rel. mRNA	Fold	Fold	Fold	Fold
VEGFA	6479.1	1.0	0.7	0.6	**0.5**
VEGFB	9685.2	1.0	1.3	1.1	2.0
VEGFC	8728.8	1.0	1.5	1.7	1.9
VEGFD	13.0	1.0	**2.1**	0.6	1.3
PGF	498.5	1.0	**0.1**	**0.1**	**0.1**
HGF	8.8	1.0	0.6	1.0	**0.3**
ANGPT1	21.4	1.0	**33.6**	**8.5**	**26.7**
ANGPT2	6172.2	1.0	**2.3**	1.4	1.3
PDGFA	22.3	1.0	**0.2**	**0.2**	**0.3**
PDGFB	2259.2	1.0	**0.3**	**0.1**	**0.4**
FGF1	1712.1	1.0	1.1	0.9	0.5
FGF2	17098.3	1.0	0.7	0.5	0.7
CXCL1	50765.8	1.0	0.8	1.0	**0.3**
CXCL2	10598.5	1.0	0.7	0.7	**0.3**
CXCL5	26460.8	1.0	1.1	**5.9**	0.9
CXCL6	3670.0	1.0	**2.8**	**2.9**	**2.1**
CXCL7	2.4	1.0	**2.0**	**6.0**	1.4
IL8	42101.0	1.0	1.0	1.2	0.6

The bold-font data indicate fold expression of >2 or <0.5 compared with the parental control. P: Parental HUVEC; R: resistant HUVEC clones; Rel mRNA: mRNA expression levels normalized using GAPD ($\times 10^3$); Fold: fold expression of cells relative to that of parental cells; VEGF: vascular endothelial growth factor; PGF: placental growth factor; HGF: hepatocyte growth factor; ANGPLT: angiopoietin; PDGF: platelet-derived growth factor; FGF: fibroblast growth factor; CXCL: chemokine (C-X-C motif) ligand; IL8: interleukin 8.

A microarray analysis of the expression levels in the resistant clones revealed that the clones had lost several vascular endothelial markers including *PECAM1/CD31, ESM1* and *HOXA9*. The decreased response to VEGF stimulation and the down-regulation of VEGFR2 expression in the resistant clones might have resulted in the down-regulation of vascular endothelial markers. A recent study demonstrated that ESM1 was detected using an ELISA at a level 3- to 10-fold higher in the sera of patients with renal cell carcinoma and the addition of the multikinase inhibitor sunitinib prevented the release of ESM1 in HUVECs when induced by VEGF in an *in vitro* study (19). Together, this evidence indicates that ESM1 might be a promising biomarker for antiangiogenic inhibitors, and we plan to evaluate ESM1 as an antiangiogenic biomarker in a clinical setting. The overexpression of the angiogenic homeobox gene HOXA9 reportedly activates the EPHB4 promoter and stimulates its expression, resulting in an increase in angiogenesis (3, 20, 21). Indeed, the microarray analysis showed that the mRNA expression levels of EPHB4 were markedly down-regulated in the resistant clones by ~1/10 of that in the parental cells (data not shown), indicating that HOXA9 and EPHB4 are involved in VEGFR2 signaling. On the other hand, we found that *CXCL5, CXCL6* and *CXCL7*, known pro-angiogenic factors, were up-regulated in resistant clones (Table II). Xu *et al*. recently reported that bevacizumab up-regulated CXCL6 expression in tumor biopsies from patients with rectal cancer 12 days after monotherapy (22). Activation of inflammatory-response pathway, such as CXCL6 up-regulation induced by VEGFR2 blockade, may involved in drug resistance or alternative activation of signaling.

Recently, the concept of endothelial-to-mesenchymal transition (endo-MT), categorized as a specialized form of epithelial-to-mesenchymal transition, has been proposed in a variety of pathologic settings, including cancer and cardiac fibrosis research (23-25). Interestingly, endo-MT- specific down-regulated genes were down-regulated in resistant clones as follows: the average expression ratios of resistant/parental cells were 0.65 for *VE-cadherin,* 0.38 for *TIE1*, 1.19 for *TIE2*, 0.01 for *PECAM/CD31*, and 0.68 for *VEGFR2*. These gene expression levels were mostly down-regulated in resistant clones, as has been observed in cells that have undergone endo-MT (data not shown). However, mesenchymal markers were not up-regulated in the resistant clones. Thus, while the loss of endothelial markers was consistent with endo-MT, a mesenchymal change was not observed in the resistant clones. This suggests that either endo-MT is not involved in drug resistance to VEGFR2-TKI or that some other factor, such as the production of TGF-β in the cancer cells, may be required for them to undergo endo-MT during the process of the development of drug resistance to VEGFR2-TKI.

Taken together, we found that clones resistant to VEGFR2-TKI exhibited a lower response to VEGF stimulation, marked down-regulation of phospho- and VEGFR2 expression occurred in two clones, and the loss of vascular endothelial markers. Our findings suggested that the mechanism of resistance to VEGFR2-TKI likely involves an escape from VEGFR2 signaling dependency and that an unknown alternative activation of signaling may be a useful therapeutic target for future cancer therapy.

Conflict of Interest Statement

No Author has any financial relationships to disclose.

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