INTRODUCTION

MAPK pathway, which consists of RAS, RAF, MAPK/extracellular signal–regulated kinase (ERK) kinase, and ERK, regulates multiple physiological and pathological events. Somatic mutations of BRAF oncogene were observed in approximately 8% of human cancers and in about 50–60% of malignant melanomas (Davies et al., 2002; Holderfield et al., 2014; Samatar and Poulikakos, 2014). A single-base substitution, V600E, is the most common activated mutation of BRAF oncogene, which activates downstream MAPK/ERK kinase1/2, ERK1/2, and a range of target proteins to promote cell proliferation and invasion (Davies et al., 2002; Holderfield et al., 2014; Samatar and Poulikakos, 2014). Three specific BRAFV600E kinase inhibitors (BRAFi)—vemurafenib (Vem), dabrafenib, and encorafenib—have been approved by Food and Drug Administration for the treatment of BRAFV600E mutated metastatic melanomas (Bollag et al., 2010; Flaherty et al., 2012; Roskoski, 2019). Although most patients showed a significant initial response and symptom relief, tumor relapse was promptly developed owing to primary or acquired resistance (Bollag et al., 2010; Flaherty et al., 2012). Molecular mechanisms of resistance to BRAFi have been illuminated by researchers, and most of them were due to the reactivation of the MAPK pathway or the activation of alternative signaling pathways (Holderfield et al., 2014; Samatar and Poulikakos, 2014). In addition to these tumor cell–autonomous mechanisms, tumor microenvironment (TME) has also shown profound effects on the development of drug resistance (Somassundram et al., 2016). It has also been reported that tumor stromal cells, such as fibroblasts (Obenauf et al., 2015), macrophages (Smith et al., 2014; Wang et al., 2015), and B cells (Somassundram et al., 2017), render tumor cells resistance to BRAFi through the secretion of cytokines/chemokines or GFs.

As a key component of TME, the tumor-associated neovascular system not only provides tumor mass with adequate angiogenesis and the evolution of BRAFV600E kinase inhibitor–acquired resistance is still poorly understood. In this study, we reported that the molecular signatures of angiogenesis were enriched in early on-treated biopsies but not in disease-progressed biopsies. The process of drug resistance development was accompanied by the remodeling of vascular morphology, which was potentially manipulated by tumor-secreted proangiogenic factors. Further transcriptomic dissection indicated that tumor-secreted IGF1 drove the vascular remodeling by activating the IGF1/IGF1R axis on endothelial cells and sustained the prompt regrowth of resistant tumor. Blockade of IGF1R with small molecules at an early stage of response disrupted vascular reconstruction and subsequently delayed tumor relapse. Our findings not only showed the correlation between IGF1-mediated tumor vascular remodeling and the development of acquired resistance to BRAFV600E kinase inhibitor but also provided a potential therapeutic strategy for the prevention of tumor relapse in clinical application.
supplies of nutrients and oxygen but also facilitates the metastatic spread of tumor cells. Tumor angiogenesis is triggered and controlled by the driver oncogenes of tumor cells. For instance, BRAF<sup>V600E</sup> mutation was reported as a driver gene to trigger a proangiogenic response by regulating the expression profile of angiogenesis-related factors in papillary thyroid carcinoma, melanoma, colorectal cancer, and myopericytoma (Bottos et al., 2012; Durante et al., 2011; Sadow et al., 2014), and tumor angiogenesis was blocked by BRAFi in xenograft models of melanoma and colon cancer (Bottos et al., 2012). Accordingly, combination regimens with BRAFi and antiangiogenesis drug bevacizumab synergistically inhibited tumor growth (Comunanza et al., 2017). However, the relationship between tumor angiogenesis and BRAFi-acquired drug resistance remains poorly understood.

In this study, we investigated the cross-talk between tumor vascularization and the evolution of acquired resistance with clinical data and a mouse model. We showed that BRAFi disrupted the integrity of tumor-associated vascularization accompanied by tumor initial shrinkage, and the vessels were reconstructed along with tumor relapse. This vascular remodeling was potentially driven by ligand IGF1. Cotargeting of IGF1R and the BRAF oncogene synergistically delayed tumor relapse by interruption of vascular reconstruction.

RESULTS

The molecular signatures of tumor angiogenesis were enriched in on-treatment biopsies during BRAF-targeted therapy

To illuminate the relationship between the evolution of acquired resistance to Vem and tumor angiogenesis, we reanalyzed the clinical transcriptomic profiling data (Kwong et al., 2015). According to the treatment status, the biopsies were divided into three groups: baseline, on-treatment (On-Tx), and disease progression (resistant). We summarized 10 tumor angiogenesis-related terms from the Molecular Signatures Database (Liberezon et al., 2015; Subramanian et al., 2005) to evaluate the alterations of tumor angiogenic signatures (Supplementary Table S1). A trend of enrichment in On-Tx group was observed (Figure 1a, left panel). To clarify this, we quantified and compared the Gene Set Variation Analysis score between baseline and On-Tx groups. The results showed that the score of the On-Tx group is higher than that in baseline in 7 of 10 terms. Statistical significances were observed in 3 of 10 terms (Figure 1a, right panel). To validate the results, CEMiTool (Russo et al., 2018), a coexpression analysis R package, was applied to explore the gene modules enriched in the On-Tx group. Modules are defined as clusters of highly interconnected genes. CEMiTool can perform Gene Set Enrichment Analysis, allowing users to visualize the alteration of individual modules in different phenotypes. Clinical transcriptomic data, including a total of 85 samples from two independent cohorts (Hugo et al., 2015; Kwong et al., 2015), were used to evaluate the coexpression modules in On-Tx biopsies (Supplementary Figure S1a and Supplementary Table S2). A total of 4,983 genes passed through the CEMiTool filter with \( P < 0.2 \), which fell into 11 modules (Figure 1b and Supplementary Figure S1b and c). Gene Set Enrichment Analyses showed that the Normalized Enrichment Scores of modules M7 and M10 were much higher in the On-Tx group than in baseline, in the disease progression, or dual drug disease progression group (Figure 1b). With analysis of Kyoto Encyclopedia of Genes and Genomes pathways and Gene Ontology biological process terms, module M7 was significantly enriched for terms associated with tumor vascular construction or extracellular matrix assembly (Figure 1c and d and Supplementary Table S2), whereas module M10 was enriched for terms related to VEGF signaling pathway, WNT signaling pathway, and events of cell survival (Supplementary Figure S1d and e). These results are in line with the previous report that extracellular matrix signaling pathway was regarded as a driver for Vem resistance in early stage (Hirata et al., 2015). To visualize the interactions between the genes in module M7 or M10, protein–protein interaction data from the STRING database (Szklarczyk et al., 2019) were used for gene network analysis. Notably, pivotal roles of melanoma-associated endothelial marker genes (Tirosh et al., 2016), such as ROBO4, CD34, and VWF, were highlighted by the module network graph of M7 (Figure 1e and Supplementary Table S2). On the contrary, this phenomenon was not observed in module M10 by parallel analysis (Supplementary Figure S1f), implying that a unique angiogenesis signaling was activated in the process of acquiring drug resistance. Besides, we also identified gene modules that were enriched in biopsies resistant to Vem (M1, M6, and M11 in Supplementary Figure S2), in dual drug disease progression (M5 in Supplementary Figure S3), or in both disease progression and dual drug disease progression (M3 and M9 in Supplementary Figure S4). With analysis of Kyoto Encyclopedia of Genes and Genomes pathways and Gene Ontology biological process, events associated with tumor development, cell cycle, and skin development were significantly enriched (Supplementary Figures S2–4). Specifically, genes such as MET, EGFR, and VEGFA, which have been reported to be promoting Vem resistance (Comunanza et al., 2017; Girotti et al., 2013; Straussman et al., 2012), were also highlighted through protein interaction analysis (Supplementary Figures S2–4).

Considering that the driver gene of tumor angiogenesis may not be enriched in a pathway pattern, we checked the expression levels of angiogenesis-related genes individually. To identify the drivers, a gene set with 144 proangiogenic genes was summarized according to previous reports (Bottos et al., 2012; Tirosh et al., 2016) (Supplementary Table S1). We found that 26 genes were clustered, and 22 of them were significantly upregulated in the On-Tx group (Figure 1f). Among those genes, RG516, RASA4, SYT15, ADCY4, VWF, PDE2A, CLDN5, ROBO4, ECSCR, IL3RA, CD34, ESAM, CD45, MMRN2, EMCN, MYCT1, LDB2, TSPAN18, and TSPAN15 have been reported as markers of melanoma endothelial cells (Tirosh et al., 2016), which endorsed the observed results we mentioned earlier. All these bioinformatics analyses suggested that the signatures of tumor angiogenesis were significantly enriched in On-Tx biopsies, which may be involved in the development of Vem resistance.

Tumor vascular remodeling accompanied with the development of Vem resistance

According to previous reports, On-Tx biopsies are defined as tumors with objective clinical responses to Vem (Kwong...
**Figure 1. Tumor angiogenesis–related molecular signatures were enriched in On-Tx biopsies treated by vemurafenib.** (a) Heatmap and box plot of tumor angiogenesis–related terms analyzed by GSVA. Data were shown as GSVA score. Baseline, On-Tx, and resistant represent clinical melanoma biopsies taken at the time points before treatment (baseline), on the treatment of vemurafenib or vemurafenib plus selumetinib (On-Tx), and after resistance to single or dual drug (resistant), respectively. Terms in red font indicate the seven enriched terms in the On-Tx group. *P < 0.05; **P < 0.01. (b) GSEAs showing the module activity on different resistance stages after vemurafenib treatment. Baseline: biopsies before treatment; On-Tx: biopsies on the treatment of vemurafenib; disease progression: biopsies resistant to vemurafenib. Dual drug disease progression: biopsies resistant to vemurafenib + selumetinib. (c, d) Overrepresentation analysis of module M7 using gene sets from the (c) KEGG database or (d) GO database. Bar graphs show the $-\log_{10}$ adjusted $P$-value of the enrichment between genes in modules. (e) Gene networks of module M7, with gene nodes highlighted. The top 10 most connected genes (hubs) are labeled and colored on the basis of their origin: if originally present in the CEMiTool module, they are indicated as Coexpression; if inserted from the interactions file, they are indicated as Interaction. The size of the node is proportional to its degree. (f) Box plot of 26 proangiogenic factors by profiling the transcriptomic data of patient biopsies. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. ECM, extracellular matrix; EGA, European Genome-phenome Archive; FPKM, fragments per kilobase of exon model per million mapped fragments; GO, Gene Ontology; GSEA, Gene Set Enrichment Analysis; GVS, Gene Set Variation Analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes; NES, Normalized Enrichment Score; On-Tx, on-treatment.
et al., 2015; Song et al., 2017). The time span of On-Tx biopsy collection is broad and nonuniform. Because tumor early response is a timing-sensitive progress, accurate time points for biopsy collection will provide a fundament for the investigation of the correlation between angiogenesis and Vem resistance. To this end, we recapitulated the clinical therapeutic process with a mouse model. SMM102, a Vem-sensitive mouse cell line (Liu et al., 2019), was subcutaneously inoculated on both flanks of C57BL/6 mice (Figure 2a and Supplementary Figure S5a and b). In the Vem-treated group, mice were treated with either Vem or 5% DMSO five times a week until the tumor volumes reached 150–300 mm³. Tumor growth curves were profiled by the determination of the tumor volumes every 3 days (Supplementary Figure S5c). On the basis of our preliminary observation, tumor shrinkage and dormancy were observed from day 6 to day 15 after the initial response (around day 3), and subsequently, the tumor began to regrow by day 18 until the mice were killed (day 27) (Supplementary Figure S5c). Consequently, tumor biopsies were collected at the indicated time points, and DMSO-treated tumors served as control (Figure 2a and b). Notably, severe hemorrhages were observed in tumors from the control group or drug resistance group (day 27) but not in tumors from the persistent or early drug resistance group (Supplementary Figure S5d and e). Immunochemistry analyses of phosphorylated protein kinase B and phosphorylated ERK in tumors with Vem treatment showed the reactivation of MAPK and phosphatidylinositol 3-kinase/protein kinase B pathway by day 27 (Supplementary Figure S5a–d). Accordingly, the evolution of Vem resistance was defined in four stages: (i) early responsive period (Vem-3), (ii) persistent period (Vem-6), (iii) early drug resistance period (Vem-18), and (iv) stable drug resistance period (Vem-27).

We then assessed the structural and morphological alterations of tumor blood vessels during the evolution of drug resistance by CD31 immunohistochemistry staining. Within tumors treated with Vem for 3 or 6 days, blood vessels appeared to be deformed or even fragmented, and the vascular integrity was lost. The vascular system began to reconstruct on day 18 and was well-restored by day 27 (Figure 2c, upper panel). To evaluate the endothelial status during the process, we detected the apoptosis of endothelial cells by TUNEL assay combined with CD31 immunohistochemistry staining. Only slight apoptosis signals were observed by day 6 (Figure 2c, lower panel), suggesting that endothelial cells remained alive. Subsequently, we evaluated the structural integrity and biofunction of the remodeled blood vessels. FITC-labeled dextran was intravenously injected into tumor-bearing mice to visualize the leaked macromolecules. The results showed that tumor blood vessels were luxuriant, and no leakage was observed in tumors of the control group (Figure 2d). With the Vem treatment for 3 and 6 days, the vascular wall became thinner, and leakage of FITC-labeled dextran was observed, indicating that the blood vessels became dysfunctional. Little leakage was observed on days 18 and 27, implying that the vascular system was reconstructed (Figure 2d and Supplementary Video S1–5). To characterize the blood vessels in TME, the area of CD31+ staining, microvessel density of healthy vessels, microvessel lumen diameter, thickness of vascular wall, and blood vessel leakage were carefully measured at each time point (Figure 2e–i). Microvessel density, microvessel lumen diameter, and thickness of vascular wall significantly decreased in tumors on day 6 compared with those in the control group. By day 18, these vascular indexes start to restore, indicating that the vessels were remedied (Figure 2f–h). In line with these results, the most significant leakage of FITC dextran, which negatively correlated with the degree of integrity, was observed on day 6 compared with those at other time points (Figure 2i). Interestingly, the restoration of the CD31+ area was delayed until day 27 (Figure 2e). One potential explanation is that endothelial cell proliferation lagged behind vascular remodeling during tumor relapse. Taken together, the development of Vem-acquired resistance is accompanied by a process of vascular remodeling.

To distinguish the vascular remodeling from angiogenesis, we included a counterpart animal model xenografted the same tumor without treatment as the control group (Figure 2a). The tumor angiogenesis feature was characterized on days 0, 3, 6, and 9 without Vem administration (Supplementary Figure S5a and b). To avoid bias, tumors with comparable size were carefully dissected (Supplementary Figure S5d) and applied to CD31 staining (Supplementary Figure S7b). Incremental CD31+ area, microvessel density, and lumen size were observed from day 0 to day 6 (Supplementary Figure S7c–e). Interestingly, drops of CD31+ area and microvessel density were observed on day 9, which were accompanied by tumor necrosis (Supplementary Figures S5d and S7c–e). Therefore, the vascular remodeling was induced by Vem treatment but not by a natural phenomenon during tumor development. We carefully calculated the area of FITC dextran outside the blood vessels, a significant increase of FITC-dextran leakage was observed in tumors of day 6 (Figure 2f). We also performed the blood vessel perfusion in the control group at the time points of days 0, 3, 6, and 9. Despite that the leakage of FITC dextran had been observed consistently, the dispersion of FITC dextran was not serious, and no variation of FITC-dextran leakage was observed from days 0 to 9 (Supplementary Figure S7f and g).

To rule out the possibility that Vem directly induced endothelial cytotoxicity, the cytostatic and antiendothelial activities of Vem were evaluated with human dermal microvascular endothelial cells (HMECs) and human umbilical vein endothelial cells (HUVECs). Cytotoxicity assays indicated that both HMECs and HUVECs were not sensitive to Vem (Supplementary Figure S8a) (half-maximal inhibitory concentration = 7.2 μM in HMEC and 2.2 μM in HUVEC vs. half-maximal inhibitory concentration = 0.55 μM in SMM102). In line with that, the phosphorylation of ERK1/2 in HMECs and HUVECs was not suppressed by Vem (Supplementary Figure S8b). Moreover, apoptosis of endothelial cells was barely detected in the Vem treatment group (Supplementary Figure S8c). On the contrary, melanoma cells responded to BRAF inhibition very well in terms of cytotoxicity, apoptosis, as well as phosphorylation levels of downstream proteins (Supplementary Figure S8a–c). Thus, Vem-induced vascular remodeling should not be directly through endothelial cells in vivo.

In view of the observations mentioned earlier, we hypothesize that (i) tumors shrank on the blockade of driver oncogenic pathway; (ii) vascular disintegration was
Figure 2. A dynamic process of tumor blood vessels accompanied by the evolution of Vem resistance. (a) Schematic diagram of the experimental design used for melanoma mouse model with or without Vem treatment. (b) Growth curves of SMM102 tumors treated by vehicle or Vem (50 mg/kg) for the indicated days (n = 4 mice, eight tumors per group). (c) Upper panel: representative images of immunohistochemistry analysis with the endothelial marker CD31 in SMM102 tumors. Lower panel: representative images of immunofluorescence analysis with CD31 (red), TUNEL (green), and DAPI (blue) of SMM102 tumors. Bar = 100 μm. (d) Representative images of tumor blood vessels (green) with FITC-labeled 2,000 kDa dextran. Upper panel: representative images of tumor blood vessels were generated from NIH/ImageJ by the Z project (×100 magnification). Middle panel: representative images of tumor blood vessels were

- **Vascular Remodeling Regulates BRAFi Resistance**
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accompanied with drug responses at instant responsive period and persistent period; and (iii) the vascular system was reconstructed along with the resistant tumor reburst, supporting the rapidly growing tumor mass with nutrition and oxygen.

**IGF1 was identified as a potential driver for tumor vascular remodeling in the early resistance period**

To investigate the potential driver gene for tumor vascular remodeling, we profiled the transcriptomic data of Vem-treated tumors. Because tumor angiogenesis was mainly stimulated by proangiogenic factors, we then focused on the alterations of proangiogenic genes. In line with the angiogenic signatures, most of the proangiogenic factors were downregulated in tumors on day 6 compared with those on day 3 or day 18 (Figure 3a). A potential explanation is that tumor cells agitated the expression of proangiogenic factors to compensate for the angiogenic restraint caused by early oncogene blockade (day 3). However, owing to the sustained blockade of oncogenes, the compensatory proangiogenic factors were suppressed (day 6) until tumor regrowth (day 18). Thus, the driver genes, which manipulate tumor vascular remodeling, should be upregulated on day 18. According to this hypothesis, we evaluated the alterations of tumor angiogenesis-related terms, which were applied in Figure 1a, by Gene Set Variation Analysis. Enrichment of terms was observed in transcriptomic data of tumors on day 18 (Figure 3b), implying that dramatic angiogenic alterations occurred during the early resistance period. Then, we identified the differentially expressed genes and differentially expressed genes of proangiogenic factors by the algorithms of kallisto (Bray et al., 2016) and sleuth (Pimentel et al., 2017) (Supplementary Table S3). A total of 27 proangiogenic genes differentially expressed from day 6 to day 18 were summarized by volcano plot (Figure 3c). Only one of the 27 genes was downregulated; others were all upregulated from day 6 to day 18 (Figure 3c and d). Importantly, the expression alterations of these 26 genes were highly coincident with the tumor growth curves and the morphological alterations of tumor blood vessels. We also observed the expression alterations of angiogenesis signatures in control tumors; we found that the angiogenesis signatures were increased, accompanied by tumor growth (Supplementary Figure S6e). Integrating the genes upregulated in the On-Tx biopsies from clinical datasets (Figure 1f), ADCY4, VWF, IGF1, and LDB2 were concurrently identified as the potential drivers of vascular remodeling in tumors of animal models and patients (Figure 3e). Among these genes, IGF1 showed the maximum variation and significance from day 6 to day 18 (Figure 3c). Thus, we focused on the investigation of IGF1 signaling in tumor vascular remodeling thereafter.

**IGF1 was secreted by tumor cells and tumor stromal cells during targeted therapy**

The protein levels of IGF1 in Vem-treated tumors were determined by immunohistochemistry staining. In line with in vivo tumor growth curves, IGF1 was downregulated on day 6 and restored by day 18 (Figure 4a and b). Consistently, the gene expression analysis showed that the IGF1 signaling pathway was also suppressed on day 6 and restored by day 18 in Vem-treated tumors (Figure 4c). Genes in the IGF1 signaling pathway (Hänzelmann et al., 2013; Liberonz et al., 2015), such as FOS, IGFB2, IGFBP5, and IGFBP7, showed similar expression trends to those of IGF1, implying the activation of the IGF1 signaling pathway (Figure 4c).

Then, we wanted to figure out the host cell of secreted IGF1 during Vem treatment. Because the alteration tendency of IGF1 is accompanied by the processes of tumor regression and relapse, tumor cells may have the potential to secret IGF1 in response to BRAF-targeted therapy. We checked the external RNA-sequencing data generated with Vem-treated melanomas. Song et al. (2017) have defined four stages of Vem resistance in melanoma cell lines: responding (D2), drug-tolerant persisters, drug-tolerant proliferating persisters, and single drug resistant. Taking advantage of these data, we checked the alteration of IGF1 levels and observed the upregulations of IGF1 at drug-tolerant proliferating persisters stage in four of six cell lines (Figure 4d). In addition to tumor cells, IGF1 could be secreted by tumor stromal cells during the targeted therapy (Obenauf et al., 2015). To illuminate whether other types of cells in TME could secrete IGF1, we evaluated the correlations between IGF1 and the markers of tumor cells or tumor stromal cells side by side. The relationships between IGF1 and the marker genes of indicated cell types, including macrophage (CD163, CD14, and CSF1R), endothelial cell (PECAM1, VWF, and CDH5), fibroblast (FAP, THY1, and DCN), B cell (CD79A, CD79B), T cell (CD2, CD3D, and CD3E), as well as melanoma cell (S100B and MITF), were evaluated with transcriptional data of our mouse model. The results indicated that IGF1 was significantly correlated with macrophage, fibroblast, and melanoma cells (Supplementary Figure S9). Interestingly, macrophage and fibroblast also showed significant correlations with melanoma cells, which supported the contribution of these two cell types to Vem resistance (Smith et al., 2014; Strassman et al., 2012; Wang et al., 2015). Recently, single-cell RNA-sequencing data were generated with a genetic mouse melanoma model undergoing BRAF-targeted therapy, which facilitates us to determine the source of IGF1 in TME (Long et al., 2019). We analyzed the expression levels of IGF1 and IGFR1 in various cell types from untreated melanomas, regressed tumors, and progressed tumors on Vem treatment. The results indicated that fibroblasts and macrophages rather than tumor cells were identified as dominant...
sources of IGF1 (Figure 4e). Notably, the alteration of IGF1 in fibroblasts and macrophages happened to show similar trends to the trends we observed in our model (Figure 4a and b). As to IGF1R, owing to the limited number of single cells, no significant alteration was observed in either tumor cells or endothelial cells, although clear tendencies were showed (Figure 4f). To investigate whether or not the IGF1R on endothelial cells was activated during the development of Vem resistance, we evaluated its phosphorylation level by intracellular phosphorylated IGF1R (p-IGF1R)/CD31 FACS analysis. Single-cell suspensions were obtained with tumor biopsies collected at the indicated time points. After CD31 and intracellular p-IGF1R staining, cells were applied to FACS analysis. The p-IGF1R levels were evaluated with CD31+ cells and were presented in histograms along with the p-IGF1R negative control cells (Figure 4g and Supplementary Figure S10). The results showed that the intensity of p-IGF1R on CD31+ cells slightly increased from day 0 to day 6 and was vigorously activated by day 18. By day 27, the p-IGF1R level decreased back to that on day 3 or day 6. The activation peak of IGF1R happened to coincide with the time of tumor vascular remodeling, implying a potential driving mechanism.

IGF1 agitated the functions of endothelial cells in vitro
Although IGF1 has been reported as a key regulator of angiogenesis (Friedrich et al., 2018), we wondered whether the alteration of the IGF1/IGF1R axis could interpret the vascular remodeling that was observed in tumors of animal models. Thus, the function of the IGF1/IGF1R axis on endothelial cells was evaluated. Western blotting results showed that picropodophyllin (PPP), an IGF1R-specific inhibitor, efficiently suppressed the IGF1-activated signaling in HUVECs and HMECs (Figure 5a and Supplementary Figure S11a). The results of cell proliferation assay showed that both HMECs and HUVECs were inhibited by PPP either in the presence or in the absence of IGF1 (Supplementary Figure S11b). Cytotoxicity of PPP was evaluated by cell apoptosis assay. The results showed that PPP only slightly induced early apoptosis of endothelial cells.
**G Xu et al.**  
Vascular Remodeling Regulates BRAFi Resistance

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**a**

Control  Vem-3  Vem-6  Vem-18  Vem-27

**b**

![](image1)

**c**

![heatmap](image2)

**d**

![](image3)

**e**

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cells (Figure 5b and Supplementary Figure S11c). We then evaluated the effects of the IGF1/IGF1R axis on the angiogenic function of endothelial cells. As expected, the results of transwell assay indicated that PPP significantly suppressed endothelial cells migration in a dose-dependent manner (Figure 5c and d and Supplementary Figure S11d and e).

However, IGF1R inhibition only significantly impaired the endothelial cell tube formation in HUVEC in vitro (Figure 5e–g, data not shown). Interestingly, IGF1 stimulated the dilation of tube diameter rather than increased tube number. Taken together, these results suggested that IGF1 predominantly promoted blood vessel organization but not endothelial cell programmed cell death.

In addition, the activation of IGF1R was also reported as a resistant mechanism for BRAF-targeted therapy (Strub et al., 2018; Villanueva et al., 2010). To validate the function of the IGF1/IGF1R axis on tumor cells, we assessed the phosphorylation level of IGF1R in isogenic pair SMM102 and SMM102R. In line with a previous report, the phosphorylation level of IGF1R was significantly upregulated in SMM102R cells on the basis of quantification data (Supplementary Figure S12a), and MTT assays also showed that SMM102R was not sensitive to Vem or selumetinib (Supplementary Figure S12b). Then we evaluated the phosphorylation level of IGF1R and proliferative roles in SMM102 or SMM102R on treatment with PPP. We found that blockade of IGF1/IGF1R inhibited the phosphorylation level of IGF1R and the long-term cell proliferation of SMM102R but not that of SMM102 parental (Supplementary Figure S12c–f).

**IGF1R inhibition delayed tumor relapse by blocking tumor vascular remodeling**

Encouraged by the observation of vascular remodeling–acquired drug resistance correlation and the functional validation of the IGF1/IGF1R axis on endothelial cells, we then assessed the therapeutic effect of combination treatment with Vem and PPP in a mouse model. Mice were treated with 5% DMSO, Vem, PPP, or Vem plus PPP when the volumes of xenograft tumors reached 150–300 mm³ (Figure 6a). Tumor growth curves were depicted by measurement of tumor volumes every 3 days (Figure 6b). As expected, the tumor responded to Vem very well initially, but prompt relapse was observed after 18 days (Figure 6b and Supplementary Figure S13a). Tumor growth was not suppressed by monotherapy of PPP, but combination therapy with Vem and PPP synergistically significantly suppressed tumor growth for up to 27 days (Figure 6b and c). In addition, a nude mouse model xenografted with WM2664, a human BRAF-mutated cell line, was also used to evaluate the therapeutic effect of this combination treatment. Consistently, the results of the tumor growth curve showed that combination therapy with Vem and PPP synergistically suppressed tumor growth for up to 42 days compared with either single drug–treated groups or the control group (Supplementary Figure S14a). These observations were confirmed by the calculation of dissected tumor weight (Supplementary Figure S14b).

Notably, in both mouse and human melanoma–xenografted models, fewer hemorrhages were observed in the tumors of the combined drug group than in other groups, implying the potential inhibition of angiogenesis (Supplementary Figures S13b and S14a). We then evaluated the tumor blood vessels by immunohistochemistry of CD31 staining. Compared with the blood vessels in the control group, healthy and intact vessels were observed in the tumors of both Vem and PPP mono-therapy groups. Whereas severe vascular structural damage was observed in tumors treated with combined drugs (Figure 6d and Supplementary Figure S14c). Furthermore, greater activation of tumor cell apoptosis was observed after combination treatment with only slight activation of endothelial cell apoptosis (Figure 6d, lower panel, and Supplementary Figure S13c and d). The vascular parameters were carefully evaluated in each group. The CD31⁺ area and microvessel densities were significantly downregulated in combined drug–treated tumors compared with those in the Vem-treated tumor (Figure 6e and f and Supplementary Figure S14d and e). Interestingly, the inhibition of IGF1R reorganized the vascular size from macrovessels to multiple microvessels (Figure 6f and Supplementary Figure S14e), which is in line with the in vitro results (Figure 5e–g). These results suggested that IGF1/IGF1R axis plays a pivotal role in the tumor vessels remodeling process. Thus, the blockade of the IGF1/IGF1R axis in tumor environment potentially delays tumor relapse during the targeted therapy by preventing vascular reconstitution (Figure 6g).

**DISCUSSION**

Drug resistance is the most challenging problem for targeted therapies in clinics. In this study, we identified a mutually beneficial process between tumor vascularization and the evolution of Vem resistance by transcriptome analysis of clinical biopsies. By taking advantage of a xenografted genetic melanoma mouse model, we found a dynamic disruption–reconstitution process of tumor blood vessels accompanied by the tumor relapse process. Transcriptomic analysis indicated that the vascular remodeling process was supported by a serial of angiogenic factors, most notably IGF1. Inhibition of IGF1/IGF1R signaling impaired endothelial cells migration and tube formation. The combination of Vem and PPP delayed the tumor rebound growth by impeding tumor vascular remodeling.

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**Figure 4. The variation of IGF1/IGF1R axis in tumor or endothelial cells during targeted therapy.** (a) Representative images of IGF1 by IHC staining in SMM102 tumors with or without Vem treatment. Images were shown as ×100 (upper panel) and ×400 (lower panel) magnification. Bar = 200 μm. (b) Bar graph showed the quantitative data of IGF1. Error bars represent the mean ± SEM, n = 4. (c) Transcriptomic profiling of genes in IGF1 signaling pathway. Data were shown as row Z-score. (d) The transcriptomic profiling of IGF1 in melanoma cell lines treated by Vem. Data were shown as Log₂ (FPKM + 1). Baseline: without drug treatment. (e, f) Violin plot and scatter diagram of scRNA-seq data showing the expression of (e) IGF1 (e) and (f) IGF1R in tumor, fibroblast, blood vessel, NK T cell, macrophage, monocyte, neutrophil, and mast cell. The gene expression is normalized. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; Mann–Whitney test. The number of cells per group is indicated below the violin plots. (g) Flow cytometry analysis for p-IGF1R expression in endothelial cells at different time points of Vem-treated tumors. D2, 2 days after Vem treatment; DTP, drug-tolerant persistor; DTPP, drug-tolerant proliferating persistor; FPKM, fragments per kilobase of exon model per million mapped fragments; IHC, immunohistochemistry; P, progressed; p-IGF1R, phosphorylated IGF1R; R, regressed; scRNA-seq, single-cell RNA-sequencing; SDR, single drug resistant; U, untreated; Vem, vemurafenib.
Figure 5. IGF1 agitated the functions of endothelial cells in vitro. (a) Western blotting indicated protein levels in HUVECs treated by the indicated inhibitors (PPP) with or without IGF1 (10 ng/ml) for 15 minutes. (b) HUVECs were treated with the indicated inhibitors for 24 hours. The concentration of IGF1 is 100 ng/ml, whereas that of Vem is 1 μM. Cell apoptosis was determined by flow cytometry and analyzed by FlowJo_V10; levels of apoptosis were indicated in...
The TME plays a vital role in tumor progression and also promotes resistance to targeted therapy in melanoma (Somasundaram et al., 2016). As a key component of TME, tumor vascularization has been shown as an essential driver for tumor progression and resistance to targeted therapy (Klemm and Joyce, 2015). Tumor vessels could apply nutrients and oxygen, which are necessary for the emergence of tumor phenotype switching and drug resistance (Arozarena and Wellbrock, 2019; Boumahdi and de Sauvage, 2020). Researchers also observed that the switching between drug-resistant slow-cycling and -proliferating melanoma cells was dependent on oxygen levels (Roesch et al., 2013). A combination of antiangiogenesis and targeted therapy has been proposed to improve the efficacy and to overcome drug resistance. Cotargeting VEGFA and BRAF could delay the acquired resistance to Vem (Comunanza et al., 2017), and clinical trials have been carried out (NCT00387751, NCT00095459, and NCT01495988; https://clinicaltrials.gov/). In this study, IGF1 was identified as the key factor for tumor vascular remodeling but not VEGFA, which was not significantly upregulated on day 18 on Vem treatment, and also VEGFA is usually a biomarker for endothelial cell proliferation and neovascularization. Our results suggested that anti-vascular remodeling rather than anti–de novo vasculogenesis should be the antiangiogenesis strategy we applied in this study. To this end, IGF1/IGF1R axis should be an optimized target.

In addition to the vascular remodeling function in the early resistance stage, IGF1/IGF1R pathway has been reported as a driver for melanoma progression and drug resistance to BRAF-targeted therapy (Obenauf et al., 2015; Strub et al., 2018; Villanueva et al., 2010). Researchers found that the serum levels of IGF1 were higher in patients with melanoma, especially in those with metastatic melanoma (Kucera et al., 2014), and they also found that IGF1 promoted the expansion of melanoma-initiating cells by activating epithelial–mesenchymal transition process (Le Coz et al., 2016). Villanueva et al. (2010) have reported that IGF1R and p-IGF1R were upregulated in Vem-resistant cell lines and clinical biopsies, and cotargeting of MAPK/ERK kinase and IGF1R could overcome drug resistance to Vem. In line with these reports, we also found that the resistant line SMM102R but not parental line SMM102 was sensitive to IGF1R inhibition. Apart from the function in tumor progression and drug resistance, IGF1 has also been reported as a proangiogenic factor (Bach, 2015). In this case, IGF1 functioned as a proangiogenic factor for tumor vascular remodeling. Our data indicated that IGF1 could promote migration and tube formation but not the proliferation of endothelial cells, which confirmed the function of IGF1 for tumor vascular remodeling. Therefore, IGF1/IGF1R drives drug tolerance of tumor cells at the resistant stage, whereas they remodel the vascular system at the early response stage. Cotargeting of BRAF and IGF1R could delay the tumor relapse in the mouse model. In our project, we have not fully elucidated the origin of IGF1. Other researchers claimed that IGF1 was mainly secreted by B cells during BRAF-targeted therapy (Somasundaram et al., 2017). However, on the basis of our results, the expression of IGF1 was more correlated with macrophages, fibroblasts, and melanoma cells. It remains ambiguous to tell which cell type is the key source of IGF1 and how they interact with each other during the evolution of drug resistance to BRAF-targeted therapy. To overcome the limitation of our project, more work should be addressed.

In conclusion, we proposed a mechanism model driven by tumor vascular remodeling in the evolution of Vem resistance. First, melanoma was regressed by BRAF-targeted therapy. The structure and integration of tumor blood vessels were destroyed without proangiogenic factors secreted from melanoma. Then, Vem early-resistant melanoma started to provide proangiogenic factors to stimulate tumor vascular remodeling, notably IGF1, and in turn, melanoma received the survival signals from tumor blood vessels for regrowth and progression. Combination treatment with Vem and PPP blocked the tumor vascular remodeling process, which delayed tumor relapse (Figure 6g). Thus, our study emphasized the importance of IGF1-driven tumor vascular remodeling during the Vem resistance evolution, which also provided a combinatorial strategy for targeted therapy either in melanoma or in other tumor types.

**MATERIALS AND METHODS**

**Cell culture**

SMM102 cell line was derived from a spontaneous tumor in a transgenic *BrafV600E/Cdkn2a−/−/Pten−/−* mouse as previously reported (Liu et al., 2019). Human skin fibroblasts HDF-4, human melanoma cell line WM2664, and HUVECs were provided by the Core Facility of West China Hospital (Chengdu, China). HMEC cell line was a gift from Yongzhang Luo at Tsinghua University (Beijing, China). All cell lines were cultured with DMEM (HyClone Laboratories, Logan, UT) supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% carbon dioxide. SMM102R is a resistant variant of SMM102 cultured with 1 μM Vem for over 30 days.

**Animal experiments**

C57BL/6 mice (female, aged 6–8 weeks) were purchased from Beijing HFK Bioscience (Beijing, China). The mice were housed and maintained under specific pathogen-free conditions in facilities and treated humanely throughout the studies. All mouse experiments in this study were performed according to the protocols approved by the Ethics Review Committee of Animal Experimentation of Sichuan University (Chengdu, China). Mice were subcutaneously injected with 1 × 10⁵ SMM102 cells, two injections per mouse. For SMM102 in vivo growth curve on Vem treatment, once tumors volumes reached 150–300 mm³, mice were treated with vehicle or Vem (50 μM) every 5 days.
Figure 6. Combination treatment with PPP and Vem delayed the relapse of melanoma. (a, b) A schematic representation of (a) drug treatment groups and (b) their effects on the growth of SMM102 tumors in C57BL/6J mice (n = 5 mice, 10 tumors per group, mean ± SD; Vem: 50 mg/kg, PPP: 10 mg/kg). **P < 0.01; ****P < 0.0001, two-way ANOVA. (c) The volumes of SMM102 tumors treated with the indicated inhibitors on day 9 or day 27 (n = 10 tumors per group; mean ± SD). ****P < 0.0001, Mann–Whitney test. (d) Upper panel: full scan images of IHC analysis with CD31 in SMM102 tumors at ×100 magnification; Bar = 500 μm. Middle panel: representative images of CD31 IHC staining at ×400 magnification; Bar = 50 μm. Lower panel: representative images of immunofluorescence analysis with CD31 (red), TUNEL (green), and DAPI (blue) of SMM102 tumors; Bar = 100 μm. Tumor endothelial cells with apoptosis were marked by a white arrowhead. (e, f) Bar graphs indicated the quantitative data for (e) CD31+ area or (f) MVD of the healthy vessel. Bars were shown as mean ± SD (n = 4). *P < 0.05; Mann–Whitney test. (g) Working model for the relationship between tumor angiogenesis and the evolution of resistance to Vem. IHC, immunohistochemistry; MVD, microvessel density; PPP, picropodophyllin; Vem, vemurafenib.
mg/kg, 5 days per week) by intraperitoneal injection. Mice were killed at different time points or when tumor volumes reached 1,000 mm³. To analyze the p-IGF1R expression in endothelial cells, tumor mass was digested with collagenase and processed into single cells suspensions. Flow cytometry analysis was used to analyze the expression of CD31 and p-IGF1R in endothelial cells during Vem treatment.

For combination therapy with Vem and PPP, once SMM102 tumors volumes reach 150–300 mm³, mice were treated with Vem (50 mg/kg, 5 days per week), PPP (10 mg/kg/day), or Vem (50 mg/kg, 5 days per week, from day 1) plus PPP (10 mg/kg/day, from day 6) by intraperitoneal injection. Tumor sizes were measured with the same caliper every 3 days, and tumor volumes were calculated using the formula 0.5 × (length × width²); mice were killed when tumor volumes reached 1,000 mm³. In addition, human-derived cell line WM2664 was used to validate the effect of combination therapy in vivo. A total of 1 × 10⁶ WM2664 cells were subcutaneously injected in the flanks of nude mice. The experiment design was in accordance with that of SMM102 mouse models.

Data availability statement

Datasets related to this article can be found at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE161430, hosted at the National Center for Biotechnology Information Gene Expression Omnibus database (GSE161430).

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization: Jj, HS; Data Curation: GX, Jyu, XH; Formal Analysis: GX, YLu; Funding Acquisition: HS; GX; Investigation: GX, Ylu, WW, Xl, XY, YB, XH, XY, YLi, RZ, CY, HC; Methodology: HS, GX, YLu, WW, Xl; Project Administration: HS, GX, YLu; Resources: GX, YLu, WW, Xl, XY, YB, XH, Jyu, YLi, RZ, CY; Software: GX, Jyu, XH, JH, JX; Supervision: Jj, HS; Validation: GX, YLu, WW, Xl; Visualization: GX, YLu; Writing - Original Draft Preparation: GX, YLu, Jya, Jj, HS; Writing - Review and Editing: GX, YLu, Jya, Jj, HS

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2021.07.162.

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Vascular Remodeling Regulates BRAFi Resistance

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