KDR Activating Mutations in Human Angiosarcomas Are Sensitive to Specific Kinase Inhibitors

Cristina R. Antonescu,1,2 Akihiko Yoshida,1 Tianhau Guo,1 Ning-En Chang,1 Lei Zhang,1 Narasimhan P. Agaram,3 Li-Xuan Qin,1 Murray F. Brennan,2 Samuel Singer,1 and Robert G. Maki4

Departments of Pathology, Epidemiology and Biostatistics, Surgery, and Medicine, Memorial Sloan-Kettering Cancer, New York, New York

Abstract
Angiosarcomas (AS) represent a heterogeneous group of malignant vascular tumors occurring not only in different anatomic locations but also in distinct clinical settings, such as radiation or associated chronic lymphedema. Although representing only 1% to 2% of soft tissue sarcomas, vascular sarcomas provide unique insight into the general process of tumor angiogenesis. However, no molecular candidates have been identified to guide a specific therapeutic intervention. By expression profiling, AS show distinct up-regulation of vascular-specific receptor tyrosine kinases, including TIE1, KDR, SNRK, TEK, and FLT1. Full sequencing of these five candidate genes identified 10% of patients harboring KDR mutations. A KDR-positive genotype was associated with strong KDR protein expression and was restricted to the breast anatomic site with or without prior exposure to radiation. Transient transfection of KDR mutants into COS-7 cells showed ligand-independent activation of the kinase, which was inhibited by specific KDR inhibitors. These data provide a basis for the activity of vascular endothelial growth factor receptor–directed therapy in the treatment of primary and radiation-induced AS. [Cancer Res 2009;69(18):7175–9]

Introduction
Defined as highly malignant proliferations of endothelial cell differentiation, angiosarcoma (AS) represent one end of a spectrum of vascular neoplasms, which vary from benign hemangiomas to less aggressive malignancies, such as epithelioid hemangioendothelioma and Kaposi sarcoma (1). Typical clinical characteristics of AS include multifocal spread, local recurrence, and early hematogenous dissemination. Even with wide excision and irradiation, local-regional recurrence is common, and metastatic disease is also frequently observed. With the development of metastatic disease, anthracyclines and taxanes are applied first line and stand out as the two classes of agents with significant activity.

Materials and Methods
Patient selection and clinicopathologic features. Forty-two samples from 39 AS patients with available paraffin and frozen tissue for molecular analysis were included in the analysis. Primary AS occurred in 22 (56%) patients, whereas the remaining 17 patients developed secondary AS, either after radiation (14 patients) or in chronically lymphedematous upper extremities after mastectomy and radiation (3 patients). The anatomic distribution included 17 (44%) in the breast/chest wall, 14 (36%) soft tissue and bone, 4 (10%) head and neck, and 4 (10%) visceral.

Transcriptional profiling for mining candidate genes. Areas of viable tumor were microdissected and adequate quality RNA was obtained in 22 (52%) samples, which were studied on the U133A Affymetrix platform (2). Hierarchical clustering was performed using GeneSpring GX 7.3.1 software, and a gene list was identified based on significant fold changes (FC) between AS versus other sarcoma types. A second statistical analysis including only the AS samples was carried out in R5 and Bioconductor.6 The expression intensities were normalized using the robust multiarray average method (3), which includes background adjustment, quantile normalization across arrays, and probe-level expression measure summarization using median polish on the log2 scale, for each probe set. Gene expression profiles were subjected to sample clustering to discover novel subtypes using hierarchical clustering with the Euclidean distance measure and the Ward joining method. The stability of the sample clusters was evaluated using repeated resampling and lococlustering frequencies (4). Expression profiles of the two clusters were compared using differential expression analysis: an empirical Bayes t test was applied to each gene (5), and a P value cutoff of 0.0001 was used to select differentially expressed genes (P ≤ 0.0001). Sample clusters were compared with clinical variables using Fisher's exact test.

Full-length sequencing of target genes selected by expression analysis. Genomic DNA was extracted from frozen tissue in all cases. Putative exonic regions for the entire human genome were broken into 500-bp segments, and specific primers were designed using Primer3. Purified PCRs were sequenced bidirectionally with M13 primer and BigDye Terminator kit v.3.1 (Applied Biosystems).

Mutation detection. Bidirectional reads and mapping tables were subjected to a QC filter, which excludes reads that have an average phred score of <10 for bases 100 to 200. Passing reads were assembled against the PTPRD reference sequence using command line Cnsed 16.0 (6). Assemblies were passed on to PolyPhred 6.02b (7) and PolyScan 3.0 (8). The lists were merged together and the putative mutation calls were normalized to “+” genomic coordinates and annotated using the Genomic Mutation Consequence Calculator (9). All mutations were confirmed by individual PCR using different primer design and direct sequencing, in parallel with sequencing of matched normal tissue DNA.

KDR protein expression validation on AS tissue microarray. An AS tissue microarray was assembled using triplicate 0.6-cm punch biopsies from all 42 tumor samples as well as 10 additional tumors. CD31 positivity supported the presence of lesional tissue. The KDR immunoreactivity (5B11; 1:125; Cell Signaling Technology, Inc.) was scored using a three-tier grading: 1+, <20% of cells positive; 2+, 20%–75% of cells; and 3+, >75% of the cells. Using this scoring method, a 3+ KDR immunoreactivity was seen in 60% of the AS, including the four KDR-mutated tumors.

Immunofluorescence and fluorescence in situ hybridization (FISH) were performed on nine tumors showing high KDR overexpression by immunohistochemistry. Immunofluorescence antibodies used included a rabbit anti–vascular endothelial growth factor receptor 2 (VEGFR2; 5B11; 1:100; Cell Signaling Technology) and a secondary Alexa Fluor 594 goat

Note: Current address for N.P. Aragam: Indiana University School of Medicine, Indianapolis, IN. Current address for A. Yoshida: University of Tokyo, Tokyo, Japan.

Requests for reprints: Cristina R. Antonescu, Department of Pathology, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10065. Phone: 212-639-5721; Fax: 212-717-3203; E-mail: antonesc@mskcc.org.

Published Online First September 1, 2009; DOI: 10.1158/0008-5472.CAN-09-2068

5 http://www.r-project.org

6 http://www.bioconductor.org
anti-rabbit IgG (1:250; Invitrogen). For FISH, the KDR probe used included overlapping bacterial artificial chromosome clones: RP11-1122L13, RP11-168J13, RP11-152O23 (BacPac resource from CHORI), and CTD-2360L14 (ResGen, Invitrogen).

**KDR-mutant transfectants and drug treatment.** The full-length cDNA of human KDR inserted in the cloning vector PCR-Blunt II-TOPO (Open Biosystems) was cut out with KpnI and NotI restriction enzymes and ligated into a pcDNA3.1-hygro (+) expression vector (Invitrogen). KDR mutations in exon 15 KDR<sup>D717V</sup> and exon 24 KDR<sup>A1065T</sup> were introduced to the wild-type sequence by site-directed mutagenesis PCR using a QuickChange II XL kit (Stratagene). COS-7 cells were transiently transfected with expression constructs encoding cDNAs for wild-type or mutant KDR and GenJet DNA Lipofectamine transfection reagent Ver. II (SignaGen Laboratories). Before harvesting, cells were starved from serum for 6 h and stimulated with...
recombinant human VEGF (rhVEGF; R&D Systems, Inc.) for 10 min. Phosphorylated and total KDR was detected with anti–phospho-VEGFR2, Tyr1175, clone 19A10 and anti-VEGFR2 antibodies (Cell Signaling Technology). Sunitinib and sorafenib were purchased commercially. KDR exon 15–transfected (KDRD717V) and KDR exon 24–transfected (KDR-A1065T) COS-7 cells were starved from serum and growth factors for 6 h. Drugs were incubated at 37°C in the absence of serum and growth factors for 90 min. VEGF (50 ng/mL) was added only to the wild-type KDR-transfected cell culture medium 10 min before harvesting.

**Results and Discussion**

AS represent a heterogeneous group of malignant vascular tumors varying by specific etiology, such as prior radiation or lymphedema. This heterogeneity in clinical presentation made us hypothesize that different molecular pathways are driving angiosarcomagenesis that merited further evaluation and used transcriptional profiling to guide the search for mutations in key angiogenesis genes. Using an U133A Affymetrix platform, the genomic profile of 22 AS was compared with a well-characterized set of 45 soft tissue sarcomas, spanning seven histologic types (10). AS tumors formed a tight genomic group by unsupervised clustering distinct from all other sarcoma types (Fig. 1A), as a result of overexpression of genes implicated in various stages of angiogenesis. Five of the top six up-regulated genes in AS were selected for full sequencing, including TIE1 (tyrosine kinase with immunoglobulin and epidermal growth factor homology domains), KDR (kinase insert domain receptor, also known as VEGFR2), SNRK (SNF-1–related kinase), TEK [TEK tyrosine kinase, endothelial (venous malformations, multiple cutaneous, and mucosal), also known as TIE2], and FLT1 (fms-related tyrosine kinase 1, also known as VEGFR1). In addition, AS tumors showed high levels of expression of genes known as endothelial markers or endothelial function, such as PECAM1 (platelet/endothelial cell adhesion molecule, also known as CD31; FC, 6.4), EphA2 (ephrin receptor A2; FC, 6.2), ANGPT2 (angiopoietin 2; FC, 4.7), ENDerb (endothelin receptor type B; FC, 4.2), PGF (platelet growth factor; FC, 4.1), Fli1 (Friend leukemia virus integration; FC, 3.6), and VWF (von Willebrand factor; FC, 3.4). In contrast, growth factor genes, such as KIT ligand (FC, −2.3), VEGFA (FC, −2.5), and VEGFB (FC, −2.5), were down-regulated in the AS compared with other sarcoma types.

In a second step, AS tumors alone were subjected to unsupervised clustering showing two distinct genomic clusters, which correlated with anatomic location and prior exposure to radiation (P < 0.001). As shown in Fig. 1B, the first group included all radiation-induced breast AS and postlymphedema AS. In contrast, all primary breast AS and five of the six bone and soft tissue AS clustered together in a second group. Random resampling of the data showed a high frequency of clustering among repeated resamples, suggesting that the two clusters are quite stable (Fig. 1B). Among the 779 genes differentially expressed between the two clusters (P < 0.001), LYN (v-yes-1 Yamaguchi sarcoma viral–related oncogene homologue) and PRK2 (protein kinase C, 0) were specifically overexpressed in the radiation-induced AS cluster, whereas FLT1 and AKT3 were overexpressed in the non–radiation-induced AS.

Four patients with breast AS showed mutations in KDR. The observed point mutations encoded three different codons, including one in the extracellular domain Ig-like C2-type 7, exon 15 D717V, two identical mutations in the transmembrane domain, exon 16 T771R, and one in the kinase domain, exon 24 A1065T. The four KDR-mutated tumors occurred in the same anatomic region (i.e., breast/chest wall), two cases each in either primary or radiation-induced AS groups. KDR-mutated tumors showed no specific correlation.

![Figure 2](https://www.aacrjournals.org/doi-fig/10.1158/0008-5472.CAN-09-2068)

**Figure 2.** Morphologic appearance and KDR expression of AS carrying KDR mutations. A, postradiation AS of breast with a KDR kinase domain A1065T mutation, showing a conventional vasoformative growth and high-grade cytology (H&E; magnification, ×200). B, high-grade postradiation breast AS, spindle cell type, harboring a D717V KDR mutation (H&E; magnification, ×200). All above tumors show strong and diffuse KDR immunoreactivity (C), whereas the KDR immunofluorescence highlights the membranous staining pattern (D).
with histologic type or grade. Both KDR exon 16 D717V mutations occurred in primary breast AS, either low or high histologic grade. As illustrated in Fig. 2, the presence of KDR mutations was associated with a wide morphologic spectrum. Regardless of morphologic growth and histologic grade, the KDR-mutant tumors uniformly expressed strong and diffuse KDR protein either by immunohistochemistry (Fig. 2C) or by immunofluorescence (Fig. 2D). No KDR copy number alterations were detected by FISH in all KDR-positive tumors by immunohistochemistry irrespective of the status of KDR genotype (data not shown).

All four patients with KDR mutations had localized disease at the time of diagnosis but developed distant metastases to a variety of sites, including bone, liver, lung, or contralateral breast. At last follow-up, two patients were dead of disease and two were alive with disease, at 18 and 53 months, respectively. Primary tumor size was a significant predictor of overall survival in a univariate analysis (P = 0.02), but not KDR mutation status, age at diagnosis, or gender.

Autophosphorylation on tyrosine of KDRD717V and KDRA1065T was detected in lysates of transiently transfected COS-7 cells, which were starved from serum for 6 hours without rhVEGF stimulation. Tyrosine activation was absent in wild-type KDR-transfected cells under the same conditions. The phosphorylation level of both KDR mutants was slightly decreased with rhVEGF stimulation 10 minutes before harvesting, in keeping with a negative feedback loop. In contrast, wild-type KDR was tyrosine phosphorylated only when rhVEGF was added to the serum-free culture medium (Fig. 3).

Decreased KDR phosphorylation of both mutant isoforms was noted with a 0.5 μmol/L of either sunitinib or sorafenib, whereas 1 μmol/L of drugs overtly abrogated the kinase activity of the mutants (Fig. 4).

Based on its central role in vasculogenesis, it is perhaps not surprising that KDR was highly expressed in AS samples both at the transcript and protein level. Importantly, the presence of KDR mutations correlated with high levels of protein expression by immunohistochemistry. Previous studies showed KDR immunoreactivity in a similar proportion of AS (65%; 22 of 34 cases; ref. 11). In contrast with a prior report of uniform strong VEGF immunexpression in AS patients (12), our transcriptional data showed low levels of VEGF ligand expressions (VEGF-A and VEGF-B). This finding is in keeping with the potential constitutive activation of KDR in AS cells independent of exogenous VEGF. These results suggest that small-molecule receptor inhibitors, such as sunitinib or sorafenib, will be more effective than other antiangiogenic compounds, such as bevacizumab, or the anthracycline or taxane systemic therapy, which is presently used as first line for advanced disease (13–17).

The data from this study support the concept that AS represent a diverse group of malignant vascular tumors, varying by clinical presentation, such as prior exposure to radiation or lymphedema. Although postradiation sarcomas are uncommon, ~40% of all radiation-induced sarcomas develop after radiotherapy for breast cancer (18, 19). The heterogeneity of AS extends to anatomic site of tumor origin, akin to fibroblasts, which show reproducible
In summary, we show that 10% of AS bear activating mutations in KDR, which encode proteins whose autophosphorylation is blocked by KDR antagonists. Of note, the KDR-positive genotype was associated with a distinct clinical presentation of AS patients in this series, occurring in the same anatomic region (i.e., breast/chest wall), either primary in the breast or in secondary radiation-induced AS. However, analysis of larger numbers of samples should clarify if KDR mutation is characteristic only for this clinical AS subset. These findings open new avenues for specific therapeutic targeting with KDR inhibitors in a tumor characterized by an aggressive clinical course and limited management options and may have implications as pertains to angiogenesis in other cancers as well.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
Received 6/5/09; accepted 7/20/09; published OnlineFirst 9/1/09.

Grant support:

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Agnes Viale and the members of the Memorial Sloan-Kettering Cancer Center Genomics Core Laboratory, Adriana Hegry from the Sequencing Beene Core, Nicole H. Moraco for obtaining clinical data, and Milagros Soto for editorial support.

References
KDR Activating Mutations in Human Angiosarcomas Are Sensitive to Specific Kinase Inhibitors


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-09-2068

Cited articles
This article cites 19 articles, 8 of which you can access for free at:
http://cancerres.aacrjournals.org/content/69/18/7175.full.html#ref-list-1

Citing articles
This article has been cited by 16 HighWire-hosted articles. Access the articles at:
/content/69/18/7175.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.