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

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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. While representing only 1–2% 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 demonstrated ligand-independent activation of the kinase, which was inhibited by specific *KDR* inhibitors. These data provide a basis for the activity of VEGFR-directed therapy in the treatment of primary and radiation-induced angiosarcoma.

Keywords

angiosarcoma; KDR; kinase inhibitors; sorafenib; sunitinib

MATERIAL 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, while the remaining 17 patients developed secondary AS, either post-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 (13). Hierarchical clustering was performed using Genespring GX 7.3.1 software and a gene list was identified based on significant fold changes between AS versus other sarcoma types. A second statistical analysis including only the AS samples was carried out in R (<http://www.r-project.org>) and Bioconductor (<http://www.bioconductor.org>). The expression intensities were normalized using the robust multiarray average method (14), 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 were evaluated using repeated resampling and co-clustering frequencies (15). Expression profiles of the two clusters were compared using differential expression analysis: an Empirical Bayes t-test was applied to each gene (16), and a p-value cutoff of 0.0001 was used to select differentially expressed genes ($p \leq 0.0001$). 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 target regions of 500 bp or less, and specific primers were designed using Primer 3. Purified PCR reactions were sequenced bidirectionally with M13 primer and Big Dye Terminator Kit v.3.1 (Applied Biosystems).

Mutation Detection

Bi-directional reads and mapping tables were subjected to a QC filter which excludes reads that have an average phred score of < 10 for bases 100–200. Passing reads were assembled against the PTPRD reference sequence using command line Consed 16.0 (17). Assemblies were passed on to Polyphred 6.02b (18) and Polyscan 3.0 (19). The lists were merged together and the putative mutation calls were normalized to '+' genomic coordinates and annotated using the Genomic Mutation Consequence Calculator (20). 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 (TMA)

An AS TMA 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 (Cell Signaling 55B11; 1:125) was scored using a 3-tier grading: 1+, $< 20\%$ of cells positive; 2+, $\geq 20\%$ but $< 75\%$; 3+, $\geq 75\%$ of the cells. Using this scoring method, a 3+ KDR immunoreactivity was seen in 60% of the AS, including the 4 KDR-mutated tumors.

Immunofluorescence (IF) and Fluorescence In Situ Hybridization (FISH): were performed on 9 tumors showing high KDR overexpression by immunohistochemistry. IF antibodies used included a rabbit anti-VEGFR2 (55B11, Cell Signaling; 1:100) and a secondary Alexa 594 goat anti-rabbit IgG (1:250; Invitrogen). For FISH the KDR probe used included overlapping BAC 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 (OpenBiosystem) was cut out with KpnI and NotI restriction enzymes and ligated into an

pCDNA3.1-hygro (+) expression vector (Invitrogen). KDR mutations in exon 15 *KDR*^{D717V} and exon 24 *KDR*^{A1065T} were introduced to the wild type sequence by site-directed mutagenesis PCR, using a QuickchangeII 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). Prior to harvesting, cells were starved from serum for 6 hours and stimulated with recombinant human (rh)VEGF (R&D Systems, Inc) for 10 minutes. Phosphorylated and total KDR was detected with anti-phospho-VEGFR2, Tyr1175, clone 19A10 and anti-VEGFR2 antibodies (Cell Signaling Technology, Inc). Sunitinib and sorafenib were purchased commercially. KDR exon 15, *KDR*^{D717V}, and exon 24, *KDR*^{A1065T}, transfected COS-7 cells were starved from serum and growth factors for 6 hours. Drugs were incubated at 37°C in the absence of serum and growth factors for 90 min. 50 ng/ml VEGF was added only to the wild type KDR transfected cell culture medium 10 minutes before harvesting.

RESULTS AND DISCUSSION

Defined as highly malignant proliferations of endothelial cell differentiation, 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 two classes of agents with significant activity.

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 to a well-characterized set of 45 soft tissue sarcomas, spanning 7 histologic types (2). 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 6 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, a.k.a. *VEGFR2*), *SNRK* (*SNF-1* related kinase), *TEK* (TEK tyrosine kinase, endothelial [venous malformations, multiple cutaneous and mucosal], a.k.a. *TIE2*), and *FLT1* (*fms-related tyrosine kinase 1*, a.k.a. *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, a.k.a. CD31, fold change[FC] 6.4); *EPHA2* (ephrin receptor A2, FC 6.2); *ANGPT2* (angiopoietin 2, FC 4.7); *ENDRB* (endothelin receptor type B, FC 4.2); *PGF* (platelet growth factor, FC 4.1); *Fli1* (*Friend leukemia virus integration*, FC 3.6); *VWF* (von Willebrand factor, FC 3.4). In contrast, growth factors genes, such as: *KIT ligand* (FC -2.3), *VEGFA* and *VEGFB* (FC -2.5 each) were down-regulated in the AS, compared to 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 post-lymphedema AS. In contrast, all primary breast AS and 5 of the 6 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-src-1 Yamaguchi sarcoma viral related oncogene homolog*) and *PRKCθ* (*protein kinase C, theta*) were specifically overexpressed in the radiation-induced AS cluster, while *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 3 different codons, including one in the extracellular domain Ig-like C2-type 7, exon 15 *T717V*, 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 with histologic type or grade. Both *KDR* exon 16 *T717V* 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 (Figs. 2D-F) or by immunofluorescence (Figs. 2G-I). No *KDR* copy number alterations were detected by FISH in all *KDR*-positive tumors by IHC, 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.

Auto-phosphorylation on tyrosine of *KDR*^{D717V} and *KDR*^{A1056T} 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 μ M of either sunitinib or sorafenib, while 1 μ M 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 demonstrated *KDR* immunoreactivity in a similar proportion of AS (3) (65%, 22/34 cases). In contrast with a prior report of uniform strong VEGF immunoexpression in AS patients (4), 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 anti-angiogenic compounds, such as bevacizumab, or the anthracycline or taxane systemic therapy, which is presently employed as first line for advanced disease (5–9).

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 post-radiation sarcomas are uncommon, nearly 40% of all radiation induced sarcomas develop after radiotherapy for breast cancer (10,11). The heterogeneity of AS extends to anatomic site of tumor origin, akin to fibroblasts, which show reproducible differences in gene expression patterns based on the fibroblast's anatomic origin (12).

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 number 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.

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Fig. 1A

Fig. 1B

Fig. 1.

Fig 1A: Heat map of unsupervised clustering of U133A genechip transcripts reveals that the AS tumors formed a tight genomic cluster (yellow branches) distinct from all other soft tissue sarcoma types (red branches).

Fig 1B: Further unsupervised clustering of AS samples showed two distinct genomic groups, based on anatomic location and prior exposure to radiation therapy. Cluster 1 (right side) includes all primary breast angiosarcoma (BREAST), visceral (VISC), head and neck (H&N) and 5 of the 6 soft tissue & bone (ST&B) AS. Cluster 2 (left side) includes all radiation-induced breast (BREAST RX) and post-lymphedema AS.

Fig 2. Morphologic appearance and KDR expression of AS carrying KDR mutations: A. Post-radiation AS of breast with a *KDR* kinase domain *A1065T* mutation, showing a conventional vasoformative growth and high grade cytology (H&E, 200x); B. High grade post-radiation breast AS, spindle cell type, harboring a *T717V KDR* mutation (H&E, 200x); all above tumors showing strong and diffuse KDR immunoreactivity (C), while the KDR immunofluorescence highlights the membranous staining pattern (D).

Fig 3. KDR^{D717V} and KDR^{A1065T} showed constitutive auto-phosphorylation on tyrosine in the absence of serum and rhVEGF for 6 hours, whereas wild type KDR was only phosphorylated after rhVEGF stimulation.

Fig. 4. Sensitivity of KDR^{D717V}, KDR^{A1056T} and wild-type KDR transfected COS-7 cells with sunitinib and sorafenib exposure for 90 minutes in the absence of serum and growth factors (0, 0.5 and 1 μM). Both mutants showed decreased phosphorylation at 0.5 μM, while kinase activity was abrogated at 1μM of either drug.