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KINASE INSERT DOMAIN RECEPTOR ACTIVATING MUTATIONS IN HUMAN ANGIOSARCOMAS ARE SENSITIVE TO SPECIFIC KINASE INHIBITORS

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ABSTRACT
Angiosarcomas represent a heterogeneous group of malignant vascular tumours 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 tumour angiogenesis. However, no molecular candidates have been identified to guide a specific therapeutic intervention. Full-sequencing of these five candidate genes identified 10% of patients harbouring Kinase insert domain receptor mutations. A Kinase insert domain receptor positive genotype was associated with strong Kinase insert domain receptor protein expression and was restricted to the breast anatomic site, with or without prior exposure to radiation. Transient transfection of Kinase insert domain receptor mutants into COS-7 cells demonstrated ligand-independent activation of the kinase, which was inhibited by specific Kinase insert domain receptor inhibitors. These data provide a basis for the activity of VEGFR-directed therapy in the treatment of primary and radiation-induced angiosarcoma.

Keywords: Angiosarcomas, Kinase insert domain receptor, kinase inhibitors, sorafenib, and sunitinib

MATERIAL AND METHODS:
PATIENT SELECTION AND CLINICO-PATHOLOGIC 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 tumour were micro dissected and adequate quality RNA was obtained in 22 (52%) samples, which were studied on the U133A Affymetrix platform. Hierarchical clustering was performed using Gene spring 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 and Bio-conductor. The expression intensities were normalized using the robust multiparty average method, 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 re-sampling and co-clustering frequencies. Expression profiles of the two clusters were compared using differential expression analysis: an Empirical Bayes t-test was applied to each gene and a p-value cut off 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 deoxyribonucleic acid was extracted from frozen tissue in all cases. Putative exotic 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 bi-directionally with M13 primer and Big Dye Terminator Kit v.3.1 (Applied Bio-systems).

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. Assemblies were passed on to Polyphred 6.02b and Polyscan 3.0. The lists were merged together and the putative mutation calls were normalized to '+' genomic coordinates and annotated using the Genomic Mutation Consequence Calculator. All mutations were confirmed by individual PCR using different primer design and direct sequencing, in parallel with sequencing of matched normal tissue deoxyribonucleic acid.

KDR PROTEIN EXPRESSION VALIDATION ON AS TISSUE MICROARRAY:

An AS Tissue Microarray was assembled using triplicate 0.6 cm punch biopsies from all 42 tumour samples, as well as 10 additional tumours. CD31 positivity supported the presence of lesional tissue. The Kinase insert domain receptor immunore activity (Cell Signalling 55B11; 1:125) was scored using a 3-tier grading: 1+, < 20% of cells positive; 2+, ≥ 20% but < 75%; 3+, ≥ 75% of the cells. Using this scoring method, a 3+ Kinase insert domain receptor immunore activity was seen in 60% of the AS, including the 4 Kinase insert domain receptor mutated tumours.

IMMUNE-FLUORESCENCE AND FLUORESCENCE IN SITU HYBRIDIZATION:

Immune-fluorescence and Fluorescence in Situ Hybridization were performed on 9 tumours showing high Kinase insert domain receptor over expression by immuno-histo-chemistry. IF antibodies used included a rabbit anti-VEGFR2

KINASE INSERTS DOMAIN RECEPTOR MUTANT TRANSFECT ANTS AND DRUG TREATMENT:

The full length deoxyribonucleic acid of human Kinase insert domain receptor inserted in the cloning vector PCR-Blunt II-TOPO (Open Bio-system) was cut out with KpnI and NotI restriction enzymes and ligated into an pCDNA3.1-hygro (+) expression vector. Kinase insert domain receptor mutations in exon 15 Kinase insert domain receptor and exon 24 Kinase insert domain receptor were introduced to the wild type sequence by site-directed mutagenesis PCR, using a Quick changeII XL kit (Stratagene). COS-7 cells were transiently transfected with expression constructs encoding DNAs for wild type or mutant Kinase insert domain receptor and GenJet deoxyribonucleic acid 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 for 10 minutes. Phosphorylated and total Kinase insert domain receptor was detected with anti-phospho-VEGFR2, Tyr1175, clone 19A10 and anti-VEGFR2 antibodies (Cell Signalling Technology, Inc). Sunitinib and sorafenib were purchased commercially. KDR exon 15, *KDR*, and exon 24, *KDR*, 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 Kinase insert domain receptor 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 neo-plasms, which vary from benign hemangiomas to less aggressive malignancies, such as epithelioid hemangioendothelioma and Kaposi sarcoma. 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 tumours 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. AS tumours formed a tight genomic group by unsupervised clustering distinct from all other sarcoma types as a result of over expression 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)*, Kinase insert domain receptor a.k.a. *VEGFR2)*, *SNRK (SNF-1 related kinase)*, tyrosine kinase, endothelial [venous malformations, multiple cutaneous and mucosal], *a.k.a. TIE2)*, and *FLT1 (fms-related tyrosine kinase 1*, a.k.a. *VEGFR1)*.

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