A Recurrent Activating PLCG1 Mutation in Cardiac Angiosarcomas Increases Apoptosis Resistance and Invasiveness of Endothelial Cells

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Abstract

Primary cardiac angiosarcomas are rare tumors with unfavorable prognosis. Pathogenic driver mutations are largely unknown. We therefore analyzed a collection of cases for genomic aberrations using SNP arrays and targeted next-generation sequencing (tNGS) of oncogenes and tumor-suppressor genes. Recurrent gains of chromosome 1q and a small region of chromosome 4 encompassing KDR and KIT were identified by SNP array analysis. Repeatedly mutated genes identified by tNGS were KDR with different nonsynonymous mutations, MLL2 with different nonsense mutations, and PLCG1 with a recurrent nonsynonymous mutation (R707Q) in the highly conserved autoinhibitory SH2 domain in three of 10 cases. PLCγ1 is usually activated by Y783 phosphorylation and activates protein kinase C and Ca2+-dependent second messengers, with effects on cellular proliferation, migration, and invasiveness. Ectopic expression of the PLCγ1-R707Q mutant in endothelial cells revealed reduced PLCγ1-Y783 phosphorylation with concomitant increased c-RAF/MEK/ERK1/2 phosphorylation, increased IP3 amounts, and increased Ca2+-dependent calcineurin activation compared with ectopic expressed PLCγ1-wild-type. Furthermore, coflin, whose activation is associated with actin skeleton reorganization, showed decreased phosphorylation, and thus activation after expression of PLCγ1-R707Q compared with PLCγ1-wild-type. At the cellular level, expression of PLCγ1-R707Q in endothelial cells had no influence on proliferation rate, but increased apoptosis resistance and migration and invasiveness in in vitro assays. Together, these findings indicate that the PLCγ1-R707Q mutation causes constitutive activation of PLCγ1 and may represent an alternative way of activation of KDR/PLCγ1 signaling besides KDR activation in angiosarcomas, with implications for VEGF/KDR targeted therapies. Cancer Res; 74(21): 1–11. © 2014 AACR.

Introduction

Primary malignant cardiac tumors are rare and encompass several sarcoma entities (1). Besides undifferentiated sarcomas angiosarcomas are most frequent. Angiosarcomas are derived from endothelial cells and encompass a heterogeneous group of tumors with variable histologic characteristics and several clinical forms besides cardiac angiosarcomas, such as the skin and scalp angiosarcomas, angiosarcomas arising in irradiated fields, and hepatic angiosarcomas related to occupational causes (2–4). Cardiac angiosarcomas are characterized by a high proliferation rate and a marked propensity to metastasize, mostly to the lung (5, 6). Patients have usually developed metastases at the time of diagnosis and prognosis is extremely adverse due to the inoperable localization and a distinct insensitivity toward chemotherapeutic radiotherapy. Because of a lack of clinical studies, the current therapy is oriented on morphologic similar sarcomas of other localizations and mainly based on the inhibition of angiogenesis (7, 8).

Knowledge regarding the pathogenesis of cardiac sarcomas is so far very limited and only studies of few or single cases of cardiac sarcomas of various entities using cytogenetics and mutational analyses of KRAS and TP53 are available (9–11). For cardiac angiosarcomas, these studies described several numerical abnormalities and KRAS mutations in 2 of 3 and p53 mutations in 2 of 4 cases analyzed (12). For angiosarcomas of other locations, recently recurrent mutations in PLCγ1 and PTPRB were detected (13).

To gain insight into the pathogenesis of primary cardiac angiosarcomas, we analyzed a collection of formalin-fixed paraffin-embedded (FFPE) biopsies of 10 cases for genomic aberrations. SNP arrays were used to detect genomic gains and losses and targeted next-generation sequencing (tNGS), which
is suitable for the analysis of DNA from FFPE biopsies, was used to detect single-nucleotide variants (SNV) and small insertions and deletions (indel) in 409 frequently mutated oncogenes and tumor-suppressor genes.

**Materials and Methods**

**Tumor samples and cell lines**

Primary cardiac angiosarcomas were collected by the Departments of Heart Surgery and Pathology, Westphalian Wilhelms-University (Münster, Germany), from 2002 to 2013. The study was conducted in accordance with the Declaration of Helsinki and approved by the local Ethics commission (Ethics commission of the Medical Faculty of the Westphalian-Wilhelms University Münster, 2009-283-f-S). HEK293 were obtained from the DSMZ (Braunschweig, Germany) and the identity was regularly (each 6 months) confirmed using short tandem repeat (STR) profiling (AmpFlSTR Profiler Plus; Life Technologies). Human umbilical vein endothelial cells (HUVEC) were obtained from PromoCell, cultured in standard endothelial cell growth medium [ECGM; containing 0.1 ng/mL EGF and 1 ng/mL basic fibroblast growth factor (bFGF); PromoCell] and used at passage 1 to 10. For stimulation of PLCγ1 ECGM with higher concentrations of growth factors (ECGM2; containing 5 ng/mL EGF, 10 ng/mL bFGF, 20 ng/mL IGF, and 0.5 ng/mL VEGF 165; PromoCell) was used. Cell numbers were counted using a hemocytometer.

**DNA extraction**

Tumor cell–rich regions (fraction of tumor cells >80%) and tumor cell–free regions were microdissected from 5-μm tissue sections of the FFPE biopsies and genomic DNA was extracted using the Maxwell 16 FFPE LEV DNA Purification Kit (Promega).

**SNP array analysis**

SNP array analysis was based on Illumina’s HumanOmniExpress-FFPE BeadChip (Illumina) adapted for DNA extracted from FFPE tissue. The procedure comprises a quantitative-PCR quality control, a FFPE DNA restoration step, and the Infinium HD FFPE assay, based on whole-genome amplification, DNA fragmentation, hybridization of fragmented DNA to 50mer probes bound to beads on the array, and enzymatic single base extension with differently labeled nucleotides to discriminate between AT and GC SNPs. Subsequently, color and signal intensity was detected using Illumina’s iScan imaging system.

Log R ratios (LRR) and B-allele frequencies (BAF) were analyzed with the Illumina Genome Studio software. LRR is the ratio between observed and expected probe intensity and informative regarding copy number alterations, while BAF is the B-allele frequency and informative regarding heterozygosity.

**Immunohistochemistry**

Immunohistochemical analyses were performed with the Dako REAL Detection System (Dako). Details for the antibodies used are given in Supplementary Table S1.

**Targeted next-generation sequencing**

For the detection of somatic mutations in 409 frequently mutated tumor-suppressor and oncogenes, massive parallel semiconductor sequencing with an Ion Torrent personal genome machine (Ion Torrent; Life Technologies) was applied. DNA (40 ng) from six FFPE primary cardiac angiosarcomas and from the corresponding normal tissue was target enriched with an initial multiplex PCR (IonTorrent AmpliSeq Comprehensive Cancer Panel) and quantified using an Agilent Bioanalyzer (Agilent Technologies). The template preparation was performed with the Ion OneTouch 200 Template Kit and the massive parallel semiconductor sequencing reaction was executed with the Ion PGM 200 Sequencing Kit. Sequence reads were aligned to Human hg19 and the analysis of the alignments was performed with the Ion Torrent Variant Caller plugin. For identification of SNPs, somatic SNV, and indels, the alignments of tumor tissue and corresponding normal tissue were compared and visualized with the Integrative Genomic Viewer (Broad Institute, Cambridge, MA).

**PCR and Sanger sequencing**

PCRs were performed with a Fast PCR System (Life Technologies) at 95°C for 2 minutes and 40 cycles at 94°C for 10 seconds and 63°C for 20 seconds. PCR products were purified with ExoSAP-IT (Affymetrix) and sequenced with the BigDye Cycle Sequencing Kit and an ABI 3730 Genetic Analyzer (Life Technologies). Primer sequences are given in Supplementary Table S2.

**Site-directed mutagenesis**

The PLCG1 expression vector (pCMV6-C-Myc-DDK) was obtained from OriGene (RC216448). The GeneArt Site-Directed Mutagenesis Kit (Life Technologies) was used to generate the mutant PLCγ1 (PLCγ1-R707Q). Primer sequences for site-directed mutagenesis are given in Supplementary Table S2.

**Transfection of HEK293 cells and HUVECs**

For transfection of HEK293 cells, the FuGENE HD Transfection Reagent (Promega), and for HUVECs, PromoFectin-HUVEC was used (PromoKine). HUVECs were seeded in 12-well plates (40,000–70,000 cells/well) and transfected with 3-μg plasmid using 6-μL PromoFectin reagent or seeded in 6-well plates (100,000–150,000 cells/well) and transfected with 6-μg plasmid using 12-μL PromoFectin. Transfection efficiencies were determined with a plasmid encoding EGFP (pcDNA3-EGFP, length 6 kb; Addgene) and flow cytometry after 24 hours, and were 72% transfected cells for HEK293 and 59% for HUVECs (Supplementary Fig. S1).

**Inhibition of protein tyrosine phosphatases**

Transfected cells were treated with 100 μmol/L pervanadate for 1 hour. A 30-mmol/L pervanadate stock solution was freshly prepared by dilution of 200-mmol/L sodium orthovanadate in PBS and addition of hydrogen peroxide to a final concentration of 0.18%. The mixture was incubated for 15
minutes at room temperature and diluted in the cell culture medium to the final concentration.

**Western blot analysis**
Whole-cell lysates were prepared by lysis of 100,000 cells in 50-µL SDS sample buffer (Life Technologies) and analyzed as previously described (14). Details for the antibodies used are given in Supplementary Table S1.

**IP-1 ELISA**
IP1 amounts in HEK293 cells were measured with the IP-One ELISA Kit (Cisbio) at 450 nm.

**Flow cytometry**
For apoptosis, detection HUVECs were stained with Annexin V–Alexa Fluor-488 and propidium iodide (PI; Life Technologies) and analyzed by flow cytometry. For cell-cycle analysis, HUVECs were fixed in 70% ethanol for 2 hours at −20°C, washed in PBS/1% BSA, followed by staining with PI/RNase Staining Buffer (Calbiochem, Merck Millipore), and analyzed by flow cytometry.

**Migration and invasion assay**
HUVECs were transfected with PLCγ1-R707Q, PLCγ1-WT, or GFP. After 24 hours, 2,500, 3,000, or 5,000 cells were seeded in a two-chamber invasion assay system in which the chambers are separated by a membrane with 8-µm pores alone or coated with a Matrigel layer (BD Biosciences). After 24 hours, the migrated or invaded cells were fixed, stained with the Differential Quik Stain Kit (Polysciences Inc.) and counted.

Figure 1. Detection of a recurrent gain encompassing KDR and KIT by SNP array analysis. A, the recurrent gain of chromosome 4 was identified by SNP array analysis using the Illumina HumanOmniExpress-FFPE Bead Chips with an overlapping region of about 2 Mb (highlighted as red box). LRR data and BAF were examined by visual inspection using the Illumina Genome Studio software. Genes within the region of gain were identified by the Illumina Karyostudio Software and the two RTKs are highlighted in red. B and C, the expression of KDR was analyzed by immunohistochemistry. Both cases with the recurrent gain of chromosome 4 and strong expression are shown in B and one case with weak expression is shown in C.
Results

Detection of a recurrent gain encompassing KDR and KIT by SNP array analysis

We performed SNP array analysis for genome-wide detection of genomic gains and losses with DNA from eight cardiac angiosarcomas using the Illumina HumanOmniExpress-FPPE BeadChip examining \( \approx 700,000 \) SNPs with a median marker spacing of 2.1 kb. Gains and losses were detected in all cases with a range of two to 15 and an average of nine aberrations per case (Supplementary Table S3). Besides repeated 1q gains, a recurrent gain of a small region of chromosome 4 in two cases (overlapping region 2 Mb) encompassing two receptor tyrosine kinase (RTK) genes, KIT and KDR (Fig. 1A), was identified. KDR and KIT are known oncogenes and we therefore performed immunohistochemistry to determine whether the genomic gains were accompanied by an increased expression (Fig. 1B). KDR was expressed in all 8 cases analyzed ranging from weak (1 case) over intermediate (4 cases) to strong (3 cases) staining, and both cases with the gain showed strong expression (Fig. 1B and C). KIT expression was investigated in 5 cases, including the two cases with the gain. However, only one of the two cases showed weak expression and KIT was not expressed in any of the other cases.

Identification of recurrent mutations in PLCG1, KDR, and MLL2 by tNGS

Because of the rarity of cardiac sarcomas, access to native tumor samples and corresponding normal tissue is limited. Usually only small amounts of FFPE biopsies are available and tNGS with an initial multiplex PCR is then a suitable approach for the detection of somatic SNVs and small indels (15). We therefore used the IonTorrent AmpliSeq Comprehensive Cancer Panel for the detection of somatic mutations in 409 frequently mutated oncogenes and tumor-suppressor genes (16,000 amplicons covering about 1.6 Mbp). Six cardiac angiosarcomas for which corresponding normal tissue was available, by Sanger sequencing for all nonsynonymous mutations identified by tNGS, one further PLCG1 mutation was detected.

Three genes were recurrently mutated, KDR, MLL2, and PLCG1. KDR, which is also frequently mutated in angiosarcomas of other locations (16), was mutated in two samples, in one case in an extracellular immunoglobulin domain (R720W), and in the other case in the transmembrane domain (T771K), where several mutations were also detected in other tumors [Catalogue of Somatic Mutations in Cancer (COSMIC), release 68; Wellcome Trust Sanger Institute, Hinxton, United Kingdom]. MLL2 was mutated in two samples and in one case two mutations were detected. Each mutation created a nonsense codon (Q1613*, R5454*, and R4904* in the 5537-amino acid–long protein), and thus most likely inactivated MLL2. In line with our results, recent studies identified loss-of-function mutations in MLL2 disseminated over the whole coding region in several cancer types, indicating that MLL2 is most likely a tumor suppressor (COSMIC; ref. 17).

A recurrent missense mutation in PLCG1 (c.2120G>A; p. R707Q) was detected in three angiosarcomas. PLCG1 possesses two SH2 domains, the N-terminal (nSH2) for binding to other proteins and the C-terminal (cSH2) involved in autoinhibition (18), and the mutated R707 is located within the evolutionary highly conserved cSH2 domain (Fig. 2). The same mutation has recently been detected in 9% of angiosarcomas of other locations (13).

The R707Q mutation activates PLCγ1

To examine the consequences of the R707Q mutation in the PLCγ1-cSH2 domain, we used a vector in which PLCγ1 expression is driven by the cytomegalovirus (CMV) promoter (PLCγ1-WT) and performed site-directed mutagenesis to generate the mutated PLCγ1 variant (PLCγ1-R707Q). PLCγ1-WT and PLCγ1-R707Q were then transiently transfected into HUVEC as angiosarcomas are derived from endothelial cells. Twenty-four hours after transfection, strong expression of both PLCγ1 forms was observed in Western blot analysis (Fig. 3A), with


Figure 2. Localization of the PLCγ1 mutation and evolutionary conservation of C-terminal SH2 domain. Schematic description of the functional domains of PLCγ1, with an expanded view of the cSH2 domain. Sequence alignment of the cSH2 domain among various species shows that the entire cSH2 domain (R707 highlighted in red) is highly conserved.
weaker expression of the PLCγ1-R707Q mutant, which was also observed in all further experiments although identical plasmid amounts from various preparations were used.

PLCγ1 is usually in an inactive state, in which the catalytic center is blocked by an intramolecular interaction with the autoinhibitory cSH2 domain. After ligand-induced RTK activation and autophosphorylation, PLCγ1 can bind via its nSH2 domain to phosphorylated RTKs, which then phosphorylate PLCγ1 at Y783. Because of subsequent intramolecular binding of phosphorylated (p)-Y783 to the PLCγ1-cSH2 domain, autoinhibition is relieved and PLCγ1 activated (19). When PLCγ1-WT- and PLCγ1-R707Q-transfected HUVECs were cultured in standard ECGM with low amounts of growth factors, a significantly decreased phosphorylation of PLCγ1-R707Q at Y783 compared with PLCγ1-WT was observed (Fig. 3A). Incubation for 5 minutes in medium with higher growth factor concentrations induced stronger increases of p-Y783 in PLCγ1-WT (60%) than in PLCγ1-R707Q (17%)–transfected cells.

The strongly reduced Y783-phosphorylation in PLCγ1-R707Q could be due to mutation-induced conformational changes preventing either Y783 phosphorylation by RTKs or intramolecular binding of p-Y783 to the cSH2 domain, which would protect p-Y783 from dephosphorylation by phosphatases. To distinguish between both possibilities, we added the protein tyrosine phosphatase inhibitor pervanadate to HUVECs 24 hours after transfection. Incubation with 100-μmol/L pervanadate for 1 hour caused significant increases of p-Y783 in PLCγ1-WT and PLCγ1-R707Q (Fig. 3B). Together, these findings suggest that the R707Q mutation causes a conformational change that does not prevent phosphorylation of Y783 by RTKs but prohibits the interaction of p-Y783 with the cSH2 domain.

As the intramolecular interaction of p-Y783 with the autoinhibitory cSH2 domain is necessary for PLCγ1-WT activation, we next analyzed whether PLCγ1-R707Q is still active. Activated PLCγ1 cleaves PIP2 to generate DAG and IP3 and DAG can activate the RAF–MEK–ERK pathway via protein kinase C and IP3 via Ca2+ release Ca2+−dependent enzymes, such as the serine/threonine phosphatase calcineurin, which dephosphorylates nuclear factor of activated T cells (NFAT) in endothelial cells (20–22). Expression of the PLCγ1-R707Q mutant in HUVECs and HEK293 cells caused stronger phosphorylation of c-Raf, MEK, and ERK1/2 than PLCγ1-WT (Fig. 4A). Furthermore, expression of PLCγ1-R707Q resulted in increased amounts of IP1, which was quantified as a surrogate for IP3 (Fig. 4B; ref. 23), and decreased NFAT phosphorylation compared with wild-type PLCγ1 (Fig. 4C).

Taken together, these observations indicate that the PLCγ1-R707Q mutation causes conformational changes that prevent intramolecular binding of p-Y783 to the autoinhibitory cSH2 domain with concomitant increased activation of DAG and IP3-dependent signaling compared with PLCγ1-WT, indicating that the R707Q mutation causes constitutive activation of PLCγ1 independent of RTKs. These experimental findings are in line with results of structure modeling for the PLCγ1-R707Q mutant, which predict a loss of autoinhibition (13).

The PLCγ1 R707Q mutation increases cell survival

Activation of the KDR–PLCγ1 pathway has been reported to increase proliferation of endothelial cells (20, 24). To analyze whether the PLCγ1-R707Q mutation influenced cellular proliferation, we expressed PLCγ1-R707Q and PLCγ1-WT in HUVECs and counted cell numbers after 24 and 48 hours. Small increases of the cell numbers in PLCγ1-R707Q–transfected cells compared with PLCγ1-WT were observed after 24 (16%) and 48 hours (28%; Fig. 5A). As potential influences of the R707Q mutation on cell numbers could become more obvious in media with reduced supplements, transfected cells were also

![Figure 3](image-url)
cultured in media with less supplement (1:9 diluted). However, although the number of control cells were halved after 48 hours compared with full media, only small increases in cell numbers of PLC\(_1\)-R707Q–transfected compared with PLC\(_1\)-WT–transfected cells were observed (9%; Fig. 5B).

To determine whether the increases in cell number were due to increased proliferation rates, we analyzed the cell-cycle distribution by quantification of the cellular DNA contents using PI. However, despite the increases in cell numbers, no differences in the fractions of cells in the G1, S, and G2 phases were observed between HUVECs transfected with PLC\(_1\)-R707Q and PLC\(_1\)-WT (Supplementary Fig. S2).

Previous studies also reported an antiapoptotic role of PLC\(_1\) (25–27), and the increased cell numbers could
therefore be due to an antiapoptotic effect of PLCγ1-R707Q. To investigate whether the R707Q mutation affects cell survival, we quantified the amount of apoptotic cells in HUVECs transfected with plasmids encoding PLCγ1-R707Q or PLCγ1-WT, and as reference for cells without any ectopic PLCγ1 expression also with a plasmid encoding GFP. A, the cell numbers were measured 24 and 48 hours after transfection and mean values calculated from three experiments. B, cells were also incubated in reduced ECGM (1:9 dilution) after transfection and cell numbers were measured after 48 hours. The mean values were calculated from three experiments. C and D, to analyze cell survival, cells were transfected and cultured for 48 hours with or without cisplatin (45 μmol/L) after 24 hours, stained with Annexin V and PI, and analyzed by flow cytometry. Numbers of live (Annexin V−/PI−), apoptotic (Annexin V+/PI−), and necrotic (Annexin V+/PI+) cells without cisplatin treatment are shown in C and with cisplatin treatment in D (mean values from three experiments). E, total cell numbers were determined 24 hours after cisplatin treatment (mean values from four experiments).

The PLCγ1 R707Q mutation increases migration and invasiveness of HUVECs

PLCγ1 is involved in the regulation of the reorganization of the cytoskeleton and migration and invasiveness of cells (28–30). For reorganization of the cytoskeleton, coflin plays an important role (31, 32). In its dephosphorylated form, coflin can bind to actin filaments causing severing with subsequent reorganization. Active PLCγ1 can influence the available amounts of coflin by hydrolysis of PIP2, with which coflin forms complexes, and the dephosphorylation of coflin by calcineurin or PKC-dependent activation of the coflin phosphatase SSH1 (32). We therefore examined the phosphorylation of coflin in HUVECs transfected with PLCγ1-R707Q and PLCγ1-WT after 24 hours. Western blot analyses revealed a decreased phosphorylation of coflin in cells that expressed PLCγ1-R707Q compared with PLCγ1-WT (Fig. 6A).
The PLCγ1 R707Q mutation increases migration and invasiveness of HUVECs. PLCγ1 is involved in the regulation of the reorganization of the cytoskeleton and migration and invasiveness of cells. To analyze the effects of the R707Q mutation on actin remodeling, migration, and invasion, PLCγ1-R707Q and PLCγ1-WT were transiently expressed in HUVECs. In addition, as reference for cells without any ectopic PLCγ1 expression, HUVECs were also transsected with a plasmid encoding GFP. A, coflin plays an important role in actin filament remodeling and is activated by dephosphorylation. Phosphorylation of coflin was analyzed 24 hours after transfection. Right, the fold change of phospho-coflin normalized to PLCγ1 expression calculated from three experiments. B and C, to analyze the effects on migration and invasiveness, we used a two-chamber invasion assay in which the chambers were separated by a membrane with 8-μm pores alone or coated with a Matrigel layer. The HUVECs were transfected with PLCγ1-R707Q, PLCγ1-WT, and as control with GFP. After 24 hours, 2,500, 3,000, or 5,000 cells were seeded in the migration and invasion chambers. The chambers were incubated for additional 24 hours. After incubation, the migrated or invaded cells were fixed, stained, and counted. Cell numbers for each single experiment are given in Supplementary Table S6. Note that the numbers of migrating and invading cells for PLCγ1-R707Q are likely underestimated, as we observed in all experiments where PLCγ1 expression was controlled by Western blot analysis a stronger expression of PLCγ1-WT, and the cell numbers were not corrected for amounts of PLCγ1-WT and PLCγ1-R707Q expressed.

To analyze the effects of the R707Q mutation on migration and invasiveness, we used transiently transsected HUVECs and a two-chamber invasion assay in which the chambers are separated by a membrane with 8-μm pores alone or coated with a Matrigel layer. Compared with PLCγ1-WT, PLCγ1-R707Q transfected cells showed increased migration and invasion (Fig. 6B and C). However, the extent of the effects varied largely between different experiments (Supplementary Table S6), and although increased migration was observed in all seven experiments and increased invasion in six of seven experiments comparing PLCγ1-R707Q with PLCγ1-WT, no statistical significance was reached. This is likely due to the very different behavior of HUVECs, which are primary cells isolated from different donors, regarding migration and invasion in individual experiments, as already the numbers of migrating and invading cells transfected with the control GFP-plasmid varied more than 4- and 15-fold, respectively, even when the same numbers of cells were used for the assay.

Taking the decreased coflin phosphorylation, the increased migration in all seven and the increased invasiveness in six of seven experiments comparing PLCγ1-R707Q and PLCγ1-WT together, we conclude that PLCγ1-R707Q increases cytoskeleton reorganization and migration and invasiveness of HUVECs.

Increased expression of PLCγ1 in primary cardiac angiosarcomas

As our functional analyses revealed that not only the PLCγ1-R707Q had effects on cellular behavior but also strong expression of PLCγ1-WT, we investigated PLCγ1 expression in the available cardiac angiosarcomas by immunohistochemistry (Fig. 7). Although the expression of PLCγ1 in endothelial cells of normal blood vessels was below the detection limit (Fig. 7A), PLCγ1 expression was observed in seven of eight angiosarcomas ranging from weak (3 cases) over intermediate (2 cases) to strong (2 cases). The three angiosarcomas with the PLCγ1-R707Q mutation showed no or weak staining (Fig. 7B) compared with PLCγ1-WT cases (Fig. 7C).

Discussion

The pathogenesis of primary cardiac angiosarcomas is largely unexplored and we therefore analyzed a collection of FFPE biopsies from 10 patients for genomic aberrations by SNP array analysis and tNGS. The SNP array analysis revealed for most cases complex karyotypes and recurrent gains of the entire long arm of chromosome 1 in 3 cases and a small region of chromosome 4 encompassing KDR and KIT in 2 cases. So far, only few cytogenetic studies of angiosarcomas of various locations have been published and gain of 1q was described for one further cardiac angiosarcoma (9, 10). KDR plays an important role in the regulation of endothelial cell proliferation and migration and is activated by mutations in about 10% of angiosarcomas of other locations (16, 33). As the cases with genomic gains encompassing KDR also showed strong KDR expression, genomic gains could besides nonsynonomous mutations be an additional way of aberrant KDR activation in angiosarcomas.

Using tNGS, nonsynonomous mutations were identified in all cases and affected 11 genes (Supplementary Table S3). Among these were the chromatin modifiers MLL2 and ASXL1, and all mutations detected in both genes in three cases were nonsense mutations, and in one case even two nonsense MLL2 mutations were detected. Loss-of-function mutations in MLL2 and ASXL1 disseminated over the whole coding region were also identified in recent studies of several cancer types (17, 34), and the detection of inactivating mutations in chromatin modifiers in 3 of 6 cases analyzed...
likely indicates that, as recently shown for several other malignancies, epigenetic dysregulation plays an important role in angiosarcoma.

Several replacement mutations were found in genes contributing to tyrosine kinase signaling. Besides mutations in the RTKs ERBB4 and FGFR1 and in RAF1, whose consequences are currently unknown, and an activating NRAS mutation, KDR and PLCG1 were repeatedly affected by mutations. KDR is one of the endothelial-specific RTK, plays an important role in angiogenesis, and is frequently activated by mutations in angiosarcomas of other locations (16, 35). Taking our SNP array and tNGS results together, KDR is also in cardiac angiosarcomas in a significant fraction of cases affected by genomic aberrations, although the consequences of the KDR mutations and genomic gains remain to be investigated.

PLCγ1 is an important second messenger of KDR signaling in endothelial cells. It binds to autophosphorylated KDR via its nSH2 domain, is phosphorylated at Y783 and p-Y783, then binds to the PLCγ1 cSH2 domain, causing a conformational change, which terminates autoinhibition (18, 19). The recurrent PLCγ1 mutation affects R707 in the evolutionary highly conserved autoinhibitory cSH2 domain. When PLCγ1-WT and the PLCγ1-R707Q mutant were ectopically expressed in cell lines, phosphorylation at Y783, most likely due to constitutive tyrosine kinase activity in the cultured cells, was significantly reduced in the PLCγ1-R707Q mutant compared with PLCγ1-WT. However, an increase of p-Y783 was observed in both, the PLCγ1-WT and the PLCγ1-R707Q mutant, when a tyrosine phosphatase inhibitor was applied, suggesting that conformational changes in PLCγ1 caused by R707Q do not prevent phosphorylation by RTKs but rather prevents intramolecular binding of p-Y783 to the cSH2 domain. Interestingly, in a study in which the regulation of PLCγ1 was analyzed by generation of various PLCγ1 mutants, replacements of other residues in the cSH2 domain also resulted in decreased phosphorylation of Y783 in the absence of phosphatase inhibitors (19).

Despite the significant decreases in the PLCγ1-WT–activating Y783-phosphorylation in PLCγ1-R707Q, the effects on activation of intracellular signaling pathways were always significantly stronger upon ectopic expression of PLCγ1-R707Q compared with ectopically expressed PLCγ1-WT. As the intramolecular interaction of the cSH2 with p-Y783 is essential for a conformational change and full activation of PLCγ1 (19), the R707Q mutation seems to cause a conformational change in PLCγ1, which causes constitutive activation independent of Y783-phosphorylation by RTKs, in line with structure modeling for PLCγ1-R707Q (13).

At the cellular level, PLCγ1-R707Q increased apoptosis resistance of HUVECs compared with PLCγ1-WT under normal culture conditions and more pronounced when an apoptosis-inducing agent was applied. In line with previous studies, ectopic expression of PLCγ1-WT also increased migration and invasiveness of HUVECs without additional RTK stimulation, and this effect, along with the cytoskeleton reorganization inducing coflin dephosphorylation, was significantly stronger upon ectopic expression of PLCγ1-R707Q (28–30). However, in contrast to previous studies in which KDR-activated PLCγ1 stimulation increased proliferation (20, 24), we observed no influence of ectopically expressed PLCγ1-R707Q or PLCγ1-WT without additional RTK stimulation on proliferation rates of the HUVEC preparations that we used. Taken together, these findings show that the R707Q mutation causes RTK-independent, constitutive PLCγ1 activation, and, together with the stronger PLCγ1 expression compared with endothelial cells in nearly all angiosarcomas, indicate that PLCγ1 plays an important role in the pathogenesis of primary cardiac angiosarcomas.

Activating mutations in most second messengers of RTKs such as K-, N- and HRAS, BRAF and PIK3CA have been known for years and are frequent in several tumor entities. Interestingly, despite the actual efforts for mutation identification in tumors, a recurrent potentially PLCγ1-activating mutation affecting the catalytic domain (S345F) has only been detected in cutaneous T-cell lymphoma (36), and the same mutation that we describe has recently also been identified in 9% of angiosarcomas of other locations (13). As the S345F mutation was neither detected in our study of cardiac angiosarcomas nor in the study of angiosarcomas of other locations, it seems that different PLCγ1 activation mechanisms are selected in different tumor entities.
In summary, our analysis indicates that epigenetic dysregulation and activated KDR/PLCγ1 signaling contribute to the pathogenesis of a significant fraction of cardiac angiosarcomas. Furthermore, we show that the R707Q mutation confers KDR-independent PLCγ1 activation and may thus cause primary resistance against VEGF/KDR-directed therapies.

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

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