Cancer Research

**DOG1 Regulates Growth and IGFBP5 in Gastrointestinal Stromal Tumors**

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**Abstract**

Gastrointestinal stromal tumors (GIST) are characterized by activating mutations of KIT or platelet-derived growth factor receptor α (PDGFRA), which can be therapeutically targeted by tyrosine kinase inhibitors (TKI) such as imatinib. Despite long-lasting responses, most patients eventually progress after TKI therapy. The calcium-dependent chloride channel DOG1 (ANO1/TMEM16A), which is strongly and specifically expressed in GIST, is used as a diagnostic marker to differentiate GIST from other sarcomas. Here, we report that loss of DOG1 expression occurs together with loss of KIT expression in a subset of GIST resistant to KIT inhibitors, and we illustrate the functional role of DOG1 in tumor growth, KIT expression, and imatinib response. Although DOG1 is a crucial regulator of chloride balance in GIST cells, we found that RNAi-mediated silencing or pharmacologic inhibition of DOG1 did not alter cell growth or KIT signaling in vitro. In contrast, DOG1 silencing delayed the growth of GIST xenografts in vivo. Expression profiling of explanted tumors after DOG1 blockade revealed a strong upregulation in the expression of insulin-like growth factor-binding protein 5 (IGFBP5), a potent antiangiogenic factor implicated in tumor suppression. Similar results were obtained after selection of imatinib-resistant DOG1- and KIT-negative cells derived from parental DOG1 and KIT-positive GIST cells, where a 5,000-fold increase in IGFBP5 mRNA transcripts were documented. In summary, our findings establish the oncogenic activity of DOG1 in GIST involving modulation of IGF/IGF receptor signaling in the tumor microenvironment through the antiangiogenic factor IGFBP5.

**Introduction**

Gastrointestinal stromal tumors (GIST) are the most common mesenchymal tumors of the gastrointestinal tract and are characterized by activating mutations in the KIT or platelet-derived growth factor receptor α (PDGFRA) genes (1-3).

Imatinib mesylate (IM) is a small molecule inhibitor of several oncogenic tyrosine kinases, including KIT and PDGFRA. About 85% of patients with metastatic GIST derive substantial clinical benefit from IM treatment; however, imatinib does not cure metastatic GIST and the majority of patients eventually progress. Second, imatinib-resistant KIT mutations within the ATP-binding and activation loop domain are commonly found in IM-resistant GIST and are believed to be the major mechanism of resistance (4-7).

The protein DOG1 (discovered on GIST-1) encoded by ANO1 (also known as TMEM16A) is a calcium-dependent chloride channel (CaCC; refs. 8-10). CaCCs are involved in diverse physiologic processes including gastrointestinal rhythmic contractions (11, 12). Notably, DOG1 was found to be highly expressed both in GIST (13) and in interstitial cells of Cajal (ICC), the putative cell-of-origin of GIST (14, 15).

In clinical practice, DOG1 is a sensitive immunohistochemical marker for GIST and is preserved in 36% of GIST that lack KIT expression or activating mutations of KIT or PDGFRA (16-18). However, DOG1 biologic functions have not been characterized in GIST. To shed light on the relevance of DOG1 for GIST tumorigenesis, we evaluated the impact of DOG1 expression and activity in various GIST models, both in vitro and in vivo.

**Materials and Methods**

**Cell lines**

GIST-T1 and GIST882 were established from human, untreated, metastatic GISTs. GIST-T1 contains a 57bp deletion in c-KIT exon 11 (19). GIST882 harbors a homozygous exon 13 missense mutation, resulting in a single amino acid substitution, K642E (20). GIST48 and GIST430 were established from GIST that had progressed, after initial clinical response, during...
IM therapy. GIST48 has a primary, homozygous exon 11 missense mutation (V560D) and a heterozygous secondary exon 17 (kinase activation loop) mutation (D820A). GIST430 has a primary heterozygous exon 11 in-frame deletion and a heterozygous secondary exon 13 missense mutation. GIST882B, GIST48B, and GIST430B are sublines, which despite retaining the activating KIT mutation in all cells, express KIT transcript and protein at essentially undetectable levels. GIST62 was derived from an untreated KIT-positive GIST with KIT exon 11 in-frame mutation, but the cell line, despite retaining the activating KIT mutation in all cells, expresses KIT transcript and protein at essentially undetectable levels (21). GIST5 and GIST474 were established from imatinib-treated GISTs, and lacked KIT expression in the primary and subsequent cultures, although they retain the KIT exon 11 mutations of the parental GIST population.

Stable short hairpin RNA transfection

Short hairpin RNA (shRNA) lentivirus for human DOG1 (NM_018043) was obtained from Sigma-Aldrich (MISSION shRNA Lentiviral Transduction Particles TRCN0000040263). GIST cells were grown to 80% confluence and then infected with 1 multiplicity of infection of either nontargeting scrambled shRNA (SHC002V) control particles or DOG1 shRNA lentiviral particles in medium containing 8 μg/mL polybrene. Fresh medium containing 4 μg/mL puromycin was added after 48 hours to select for puromycin-resistant cells.

Reagents and antibodies

Imatinib mesylate (IM) was purchased from Selleck Chemicals. 17-N-Allylamino-17-demethoxygeldanamycin (17-AAG) was purchased from Calbiochem (Merck). A rabbit polyclonal antibody against KIT was from DAKO and a monoclonal rabbit antibody against DOG1 was from Diagnostic BioSystems. Polyclonal rabbit antibodies for phospho-KIT Y703 were from Cell Signaling. β-actin antibody was purchased from Sigma-Aldrich.

In vitro assays

BrdUrd incorporation assay. Cells were incubated with 1 mM BrdUrd for 2.5 hours (GIST-T1) or 24 hours (GIST882) at 37°C and processed using the fluorescein isothiocyanate (FITC) BrdUrd Flow Kit (BD Biosciences) following the manufacturer’s instructions. Briefly, 1.5 × 10⁶ trypsinized cells were fixed, permeabilized, and digested with DNase. Cells were then stained with FITC-conjugated anti-BrdUrd and 7-amino-actinomycin followed immediately by flow cytometric analysis. Ten thousand events of each sample were acquired on a Beckman Coulter FC500 Flow Cytometer.

Sulfurhodamine B. The sulfurhodamine B (SRB) assay was used according to the method of Skene and colleagues (22). Cells were plated in 96-well flat-bottomed plates. After 24 hours, culture medium was replaced with fresh medium (with or without respective drugs) in triplicate or quadruplicate cultures. At the end of drug exposure (72 hours), cells were fixed for 1 hour and stained with 0.4% SRB (Sigma Aldrich), and the optical density was detected at 560 nm. Each experiment was repeated 3 times and figures depict a representative result.

Whole-cell patch-clamp. Whole-cell membrane currents were recorded in GIST-T1 and GIST882 cell lines. The extracellular (bath) solution had the following composition: 150 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, 10 mM mannitol, 10 mM Na-HEPES (pH = 7.4). The pipette (intracellular) solution contained 130 mM CsCl, 10 mM NaEGTA, 1 mM MgCl₂, 10 mM LiCl, 1 mM ATP (pH 7.4) plus CaCl₂ to obtain the desired free Ca²⁺ concentration: 8 mM/L for 305 mM/L (calculated with Patchers’s Power Tool developed by Dr. Francisco Mendes and Franz Wurriehausen, Max Planck Institute for Biophysical Chemistry, Gottingen, Germany).

During experiments, the membrane capacitance and series resistance were analogically compensated using the circuitry provided by the EPC7 patch-clamp amplifier. The usual protocol for stimulation consisted of 600 ms long voltage steps from −100 to 100 mV in 20 mV increments starting from a holding potential of −60 mV. The waiting time between steps was 4 seconds. Membrane currents were filtered at 1 kHz and digitized at 5 kHz with an ITC-16 (Instrutech) AD/DA converter. Data were analyzed using the Igor software (WaveMetrics) supplemented by custom software kindly provided by Dr. Oscar Moran (Istituto di biofisica, Genova, Italy).

Western blotting. Whole-cell protein lysates were prepared from cell line monolayers according to standard protocols (23). Protein concentrations were determined with the Bio-Rad Protein Assay (Bio-Rad Laboratories). Proteins were separated by SDS/PAGE as described by Laemmli and colleagues (24) and transferred to Hybond-P membranes (Amersham Pharmacia Biotech). Changes in protein expression and phosphorylation were visualized by chemiluminescence (ECL chemi-luminescent reagent, Amersham Pharmacia Biotech) were captured and quantified using a FUJI LAS3000 system with Science Lab 2001 ImageGauge 4.0 software (Fujifilm Medial Systems).

In vivo studies

Tumor growth in vivo was evaluated by subcutaneously injecting the rear flanks of 6- to 8-week-old female athymic nude mice (NMRI nu/nu) with 10 million cells per flank transplanted with scrambled or DOG1 shRNA. Tumor growth was monitored biweekly with a caliper, and tumor volumes were calculated by [(length × width²)/2]. The experiment was stopped, mice were sacrificed, and tumors were harvested when controls reached approximately 1 cm³. Statistical analysis of the mean tumor volumes was done by pairwise comparison using one-tailed homoscedastic t test analysis.

Immunohistochemical staining

Four micrometer sections of paraffin-embedded tissues of xenograft samples were cut and mounted onto SuperFrost Plus coated slides (Langenbrinck) for immunohistochemical staining. Heat-induced antigen retrieval (waterbath) was carried out with Target Retrieval Solution Citrate buffer (Dako) at pH 6.0 or HIER T-EDTA buffer (Zytomed Systems) at pH 9.0. Specimens were stained with a monoclonal rabbit anti-DOG1...
[immunoglobulin G (IgG); clone, SP31; dilution, 1:100; 20 minutes, pH 6.0. Zytomed Systems, No. 504–3315], a monoclonal anti-Ki-67 (IgG1; clone, K-2; dilution, 1:2000; 30 minutes, pH 6.0. Zytomed Systems, No. MSK018), and a polyclonal anti-DOG1 antibody (dilution 1:200; 20 minutes, pH 9.0. Zytomed Systems, No. RP063) together with a highly sensitive and specific polymer detection system using horseradish peroxidase (ZytoChem-Plus HRP Polymer Kit, Zytomed Systems). The process for development was conducted using a permanent brown chromogenic substrate system (Permanent AEC Kit, Zytomed Systems). Finally, nuclei were counterstained with hematoxylin for 5 minutes.

RNA isolation and microarray gene expression profiling

Total RNA of cell line monolayers and tumor samples were isolated using the RNeasy Mini Kit (Qiagen), according to the manufacturer’s instructions. Residual traces of genomic DNA were removed with DNase I (Qiagen). RNA concentration and purity were determined photometrically (NanoDrop, Thermo scientific). Preparation of cRNA targets (5 µg total RNA), fragmentation, hybridization of HG-U133 plus 2.0 microarrays (Affymetrix), washing, staining, and scanning were conducted according to manufacturer’s protocols (Affymetrix) by the BioChip-Labor (Dr. Klein-Hitpass, Institute for Cell Biology, University of Duisburg-Essen, Essen, Germany). Signal intensities and detection calls were determined using Affymetrix microarray suite, version 5.0. Comparison files were further filtered to detect differentially expressed genes.

Quantitative real-time PCR

RNA extraction was conducted as described above, and cDNA synthesis was used to rank the difference in reads for each transcript between samples. A weighted score, described by the following expression:

\[ S_k = \sqrt{X_k} \times \log_{10} \left[ \frac{\max(X_k, Y_k, 1000)}{\max(X_k, Y_k, 1000)} \right] \]

was used to rank the difference in reads for each transcript between samples.

Published OnlineFirst April 10, 2013; DOI: 10.1158/0008-5472.CAN-12-3839
Results

**KIT and DOG1 are coexpressed in KIT-positive versus KIT-negative GIST cell lines**

Whole transcriptome sequencing analyses of KIT-positive parental KIT cell lines and KIT-negative sublines showed a 47- to 157-fold reduction of KIT and a 7- to 77-fold reduction of DOG1 sequencing counts, suggestive of a coregulation (Fig. 1A). Immunoblot studies confirm this observation with an 83- to 99-fold reduction of DOG1 protein levels in KIT-negative GISTs (Fig. 1B). Direct (IM) or indirect (17-AAG) pharmacologic inhibition of KIT did not abolish DOG1 expression (Fig. 1C).

**DOG1 knockdown does not affect KIT expression, cell proliferation, or IM sensitivity of GIST cells in vitro**

To investigate the biologic role of DOG1 in GIST, GIST-T1 and GIST882 cells were transduced with lentiviral particles...
We next investigated the effect of DOG1 knockdown on GIST xenografts in nude mice. DOG1 knockdown inhibits growth of GIST xenografts in these findings argue against a cell-autonomous activity of DOG1 in GIST biology.

Comparison of microarray data with qRT-PCR data

We conducted a literature search on the 40 top-ranking, differentially expressed genes. Genes that might have an impact on proliferation or apoptosis were selected for qRT-PCR validation. Among these candidates, a substantial (>3-
Figure 4. In vivo growth of DOG1 knockdown xenografts. A, Western blot analyses of DOG1 and KIT expression in GIST-T1 and GIST882 xenografts. B, immunohistochemical analysis of GIST-T1 and GIST882 xenografts. Samples were stained with hematoxylin and eosin (H&E) and with antibodies against DOG1, KIT, and Ki-67. C, tumor volume over time in nude mice implanted with GIST-T1, GIST882, and GIST430 cells after shRNA-mediated DOG1 suppression compared with scrambled shRNA controls. *P = 0.003.
fold) difference in expression levels was found for \( \text{DOG1} \) and \( \text{IGFBP5} \), whereas levels of other candidates were only marginally changed (Fig. 6A). \( \text{IGFBP5} \) levels decreased in GIST882 upon DOG1 knockdown (Fig. 6B). In DOG1-negative GIST882B, \( \text{IGFBP5} \) transcript counts were unchanged compared with the parental DOG1-positive GIST882 cell line. Notably, DOG1-negative GIST430B cell line showed \( \text{IGFBP5} \) transcriptome sequencing counts that were 5,000-fold higher than that in the parental DOG1-positive GIST430 cell line (Fig. 6C). The Ingenuity Pathway Analysis suggested the IGF pathway together with paxillin signaling as the top-ranking pathways affected by the DOG1 knockdown.

**Discussion**

With the introduction of imatinib, a potent KIT inhibitor, the treatment of GIST as a mainly KIT/PDGFRα-driven sarcoma has been revolutionized. Most patients whose tumors harbor activating KIT mutations benefit from IM-treatment with a median progression-free survival of 12 to 24 months (27). Although a subset of patients remains free of progression, the majority eventually progress, at which point their therapeutic options are limited. Novel treatment strategies to prevent or overcome resistance are therefore urgently needed.

DOG1 (Ano1/TMEM16A), a CaCC, was identified in microarray studies among a number of genes whose expression was significantly higher in GIST than in other soft tissue sarcomas (13, 28). It has since then proven to be a reliable immunohistochemical marker in pathologic practice (17). Mutations of \( \text{DOG1} \) have not been found in GIST (29) but expression levels are also high in ICC, the cells thought to share a common progenitor with GIST (14, 15). Whether DOG1 is a lineage-specific marker that plays a role in differentiation or also might play a transforming role in GIST is yet unclear.

To our knowledge, we are the first to investigate DOG1 biologic roles and to assess DOG1 relevance as therapeutic target in GIST.

Pathologic and genetic analyses from resected metastases progressing on imatinib have revealed secondary KIT mutations as a common mechanism of resistance. In a subset of patients, GIST metastases have lost KIT expression, indicating that KIT oncogenic programs have been supplanted by yet unidentified alternative oncogenic drivers (30). We observed a similar phenomenon in sublines of several GIST cell lines, which lost KIT expression during cultivation ex vivo. Interestingly, these cell lines also lost DOG1 expression suggesting interdependence of expression. Notably, the majority of KIT-negative GIST tumors (64%) in clinical practice also do not express DOG1 (17).

In the studies reported herein, DOG1 knockdown resulted in strong functional inhibition of chloride currents but did not affect expression or activation of KIT and KIT-dependent signaling pathways (Fig. 2A; data not shown). In our models, DOG1 therefore does not act as key regulator of KIT, and our studies suggest that DOG1 inhibition will not synergize with KIT kinase–inhibitor drugs in inactivating KIT. Whether DOG1 expression is directly coregulated with KIT is yet unknown; however, biochemical inhibition of KIT does not affect DOG1 expression (Fig. 1C) in vitro. We further showed that neither DOG1 knockdown nor biochemical inhibitors of DOG1 alter the growth modulating effect of imatinib in vitro. However, we observed a substantial growth delay when GIST-T1 and GIST430 were grown as xenografts compared with scrambled controls in nude mice. This effect was not seen in GIST882.

DOG1 is expressed in many organs (e.g., salivary glands) and DOG1 knockout mice die soon after birth (10, 31, 32). Little is known about DOG1 expression in human tissues but the UniGene database suggests an expression pattern similar to that in mice (33). Nonetheless, DOG1 expression is still remarkably high in both GIST and ICCs compared with non-GIST sarcomas (13, 16). Notably, Stanich and colleagues showed growth inhibitory effects after DOG1 knockdown and DOG1
biochemical inhibition in ICC short-term cultures (34). They concluded that regulation of proliferation by DOG1 is related to its function as a Cl⁻ entry pathway by reducing the Cl⁻ concentration in the culture media. In contrast to Stanich and colleagues, we did not observe a reduction of phosphorylated retinoblastoma tumor suppressor protein (Rb; data not shown), as a possible explanation for a cell-cycle arrest (35).

DOG1 has also been linked to other types of cancers. Amplification of the chromosomal band 11q13, the genomic
region containing DOG1, is frequently seen in breast, bladder, head and neck, and esophageal cancer (36). Patients with squamous cell carcinomas of the head and neck (SCCHN) harboring 11q13 amplifications were associated with a poor prognosis. These findings were recently confirmed by functional studies in SCCHN cell lines that showed that DOG1 amplification was associated with increased spreading, detachment, and invasion of tumor cells (37). Similar to our findings, Duvvuri and colleagues observed inhibition of tumor growth in SCCHN xenografts after knockdown of DOG1 (38). Notably, this effect was also seen in vitro.

We did not find published evidence that provides simple answers as to why a DOG1 knockdown would affect cell growth in vitro but not in vivo, as seen in our models. We speculate that the more complex cell–cell interaction in 3-dimensional tumors compared with monolayers as well as a tumor/host interaction (e.g., vasculature) may be responsible for this effect.

Using gene expression analyses, we investigated differentially expressed genes that are directly associated with proliferation or survival. Pathway analyses using Ingenuity software suggested IGF- and paxillin signaling as the most relevant pathways affected by DOG1 knockdown. Although the change of expression levels of genes involved in the paxillin pathway was subtle, IGFBP5 was the strongest differentially expressed gene besides DOG1 as confirmed by qRT-PCR. In line with these findings, the GIST cell line GIST430B, a KIT-negative, and DOG1-negative subline of imatinib-resistant GIST430, showed a 5,000-fold increase of IGFBP5 transcripts compared with its parental DOG1-positive GIST430 cell line. IGFBP5 was not upregulated in GIST882 following DOG1 knockdown, and no growth delay was observed in vivo suggesting that IGFBP5 may not be relevant to all GIST.

IGFBP5 is one of the 6 IGFBP family members and is dysregulated in diverse types of cancer including breast cancer (39), ovarian cancer (40), and Rb (41). IGFBP5 overexpression results in ‘trapping’ of IGF1 and IGF2 with subsequent inhibition of the IGF axis pathway (42). Of note, IGFBP5 has recently been shown to suppress tumor growth and metastasis of human osteosarcomas (43). In another model, IGFBP5 overexpression prevented tumor growth by inhibition of tumor vascularity, which might explain the different biologic outcomes we found between our in vivo and in vitro experiments. Interestingly, IGF2 expression has recently been shown to predict a high mitotic index correlating with outcome in GIST.

Our results highlight the functional relevance of DOG1 in a subset of GIST and suggest that further studies are warranted to better understand DOG1 and IGF1 axis growth regulation roles in GIST.

Disclosure of Potential Conflicts of Interest

M. Schuler has commercial research grant, has honoraria from speakers’ bureau, and is a consultant/advisory board member of Novartis. S. Bauer has honoraria from speakers’ bureau from GIST. No potential conflicts of interest were disclosed by the other authors.

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Acknowledgments

The authors sincerely thank the expert technical assistance received from Miriam Backs and Julia Kettler.

Grant Support

This work was supported by funding from Max Eder Fellowship from the Deutsche Krebshilfe (S. Bauer), the Life Raft Group Research Initiative (S. Bauer and J.A. Fletcher), and NIH GI SPORE 1P50CA12703-05. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Received October 4, 2012; revised February 15, 2013; accepted March 20, 2013; published OnlineFirst April 10, 2013.

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doi:10.1158/0008-5472.CAN-12-3839

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