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ROR2 is a novel prognostic biomarker and a potential therapeutic target in leiomyosarcoma and gastrointestinal stromal tumour

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Abstract

Soft-tissue sarcomas are a group of malignant tumours whose clinical management is complicated by morphological heterogeneity, inadequate molecular markers and limited therapeutic options. Receptor tyrosine kinases (RTKs) have been shown to play important roles in cancer, both as therapeutic targets and as prognostic biomarkers. An initial screen of gene expression data for 48 RTKs in 148 sarcomas showed that ROR2 was expressed in a subset of leiomyosarcoma (LMS), gastrointestinal stromal tumour (GIST) and desmoid-type fibromatosis (DTF). This was further confirmed by immunohistochemistry (IHC) on 573 tissue samples from 59 sarcoma tumour types. Here we provide evidence that ROR2 expression plays a role in the invasive abilities of LMS and GIST cells in vitro. We also show that knockdown of ROR2 significantly reduces tumour mass in vivo using a xenotransplantation model of LMS. Lastly, we show that ROR2 expression, as measured by IHC, predicts poor clinical outcome in patients with LMS and GIST, although it was not independent of other clinico-pathological features in a multivariate analysis, and that ROR2 expression is maintained between primary tumours and their metastases. Together, these results show that ROR2 is a useful prognostic indicator in the clinical management of these soft-tissue sarcomas and may represent a novel therapeutic target.

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INTRODUCTION

Sarcomas are a heterogeneous group of over 60 tumor types which originate from mesenchymal cells and which account for approximately 1% of all human malignancies. Most sarcomas demonstrate a propensity for locally aggressive growth and distant hematogenous spread.¹⁴

Leiomyosarcomas (LMS) are malignant tumors of the smooth muscle that show a high degree of molecular heterogeneity and that are characterized by local recurrence and metastasis; currently, there exist no targeted therapies for LMS. Gastrointestinal stromal tumors (GIST) are thought to arise from the interstitial cells of Cajal in the wall of the gastrointestinal tract and have been shown to respond favorably to treatment with the tyrosine kinase inhibitor imatinib and other small molecule drugs; however, almost all GIST patients eventually develop resistance to treatment, thereby making the exploration of additional therapeutic approaches necessary.^{15, 16}

Receptor tyrosine kinases (RTKs) are a family of cell surface receptors that regulate a range of normal cellular processes through ligand-controlled tyrosine kinase activity. Over the past 20 years, deregulation of RTKs has been shown to play critical roles in cancer development and progression. RTKs are now recognized as prognostic molecular biomarkers and as targets of several oncology therapeutics.¹

ROR2 (originally named the “receptor tyrosine kinase-like orphan receptor”) is a membrane-bound RTK that is activated by non-canonical Wnt signaling through its association with the Wnt5A glycoprotein during the course of normal bone and cartilage development. ROR2 expression is required to mediate the migration of cells during palate development in mammals, and mutations in the *ROR2* gene have been shown to cause diseases such as brachydactyly type B and autosomal recessive Robinow syndrome.^{2,3}

Recently, ROR2 has been shown to have pro-tumorigenic effects in osteosarcoma, renal cell carcinoma (RCC), and melanoma cell lines using *in vitro* and xenograft experiments.⁴⁻⁶ However, the functional and prognostic roles of ROR2 in soft-tissue sarcomas have yet to be elucidated. Here, we provide evidence that the invasive abilities of LMS and GIST cells depend on ROR2 expression and, using TMAs containing tumor samples with known clinical outcome, we show that high ROR2 expression in LMS and GIST is significantly associated with poor prognosis. Taken together, these results suggest that ROR2 is a novel prognostic biomarker and potential therapeutic target in sarcomas.

RESULTS

ROR2 mRNA and protein expression in soft-tissue tumors

To determine the differences in expression of RTKs in soft-tissue sarcomas, we analyzed the mRNA transcript levels of 48 RTK genes using microarray expression data from 148 tumors. We identified *ROR2* as a gene that was undetectable in the majority of sarcoma subtypes analyzed, but that showed high levels of expression in a subset of desmoid-type

fibromatosis (DTF), leiomyosarcoma (LMS), and gastrointestinal stromal tumor (GIST) cases (Fig. S1, Tables S2 and S3).

To confirm *ROR2* expression, we performed an immunohistochemistry (IHC) study using TMAs that contained 573 soft-tissue sarcomas and benign soft-tissue tumors; these 573 cases did not overlap with the 148 cases used for gene expression profiling. Similar to our gene array findings, most tumor types on the TMAs failed to react with the *ROR2* antibody, but a significant subset of LMS, GIST, and DTF cases showed strong IHC reactivity (Table S1). Representative stains scored as strongly positive, weakly positive, or negative are shown in Fig. 1. Many tumor samples showed staining of the cytoplasmic membrane, consistent with the predicted localization of *ROR2*; in some, the membrane staining was obscured by a strong staining of the cytoplasm. Given the IHC reactivity observed in LMS, GIST, and DTF, we focused the remainder of our study on examining the role of *ROR2* expression in these tumor types.

ROR2 expression mediates the invasive abilities of LMS and GIST cells

We sought to test the hypothesis that *ROR2* expression might mediate an aggressive tumor phenotype in sarcoma cells using a series of RNA interference experiments. Three human soft-tissue sarcoma cell lines were used: LMS04, derived from a retroperitoneal lesion that had spread from a primary uterine leiomyosarcoma, LMS05, derived from a primary thigh LMS arising in the lung, and GIST48, derived from a primary GIST. Both LMS05 and GIST48 exhibited robust staining of the cytoplasmic membrane for *ROR2* protein and detectable *ROR2* mRNA expression, whereas *ROR2* expression was undetectable in LMS04 by IHC and qRT-PCR (Fig. 2A). LMS05 and GIST48 cells transfected with pooled siRNAs targeting *ROR2* (siROR2) showed approximately a 70% reduction in *ROR2* mRNA levels 48h after transfection whereas cells treated with control non-targeting siRNAs (siNT) showed no reduction in *ROR2* expression; *ROR2* levels remained undetectable in LMS04 upon siNT and siROR2 treatment (Fig. 2B).

LMS04, LMS05, and GIST48 cells treated with siROR2 or siNT were then seeded in matrigel invasion chambers and allowed to migrate through the matrix for 24h before being fixed, stained, and counted. *ROR2*-positive LMS05 and GIST48 cells showed an approximate 50% reduction in their invasive ability upon siROR2 treatment as compared with siNT treatment. No differences in invasive ability were observed in *ROR2*-negative LMS04 when treated with siROR2 or siNT (Fig. 2C). Cell viability was not significantly different between siROR2 and siNT treatment (Fig. S3). Treatment with *ROR2*-ligand Wnt5A resulted in a significant increase in invasion of the *ROR2*-positive LMS05 and GIST48 cells, an effect which was abrogated by siROR2 treatment. In contrast, *ROR2*-negative LMS04 showed no significant response to Wnt5A treatment (Fig. 2D).

Prognostic Significance of ROR2 Expression in LMS, GIST, and DTF

The inhibitory effect on *in vitro* tumor invasion in LMS and GIST cells is similar to that reported in other tumor cell lines, including those derived from osteosarcoma, melanoma, and RCC.⁴⁻⁶ To determine whether *ROR2* expression correlated with patient survival, we used IHC for *ROR2* on TMAs with clinical follow-up data. These TMAs were comprised of

147 LMS, 410 GIST, and 90 DTF cases (Table 1). The clinico-pathologic features of these cases have been published previously and are briefly described below.^{9–12}

The LMS cases consisted of 74 gynecological LMS (GYN-LMS) and 73 non-gynecological soft-tissue LMS (ST-LMS); the clinical outcome data available for each patient was disease-specific survival (DSS) and the median follow-up time was 3.1 years. None of the LMS patients had received neoadjuvant treatment in the form of chemotherapy and/or radiotherapy. For the 410 GIST cases, the available outcome data for each patient was overall survival and the follow-up period was up to 20 years from the time of diagnosis. Only four of the GIST patients analyzed had received imatinib treatment during the follow-up period. For the 90 DTF cases, the available clinical outcome data for each case was the time to disease recurrence and the median follow-up period for patients that did not have a recurrence was 5.85 years (range: 0.23yr to 20.62yr). For each of the three tumor types, the associated clinical outcome data was used to generate Kaplan-Meier curves and to calculate log-rank (Mantel-Cox) tests to determine whether survival was significantly affected in patients whose tumors expressed ROR2. In addition, a hazard ratio (HR) and its associated 95% confidence interval (CI) were calculated to quantify the effect of ROR2 expression on patient outcomes. For all analyses, cases staining strongly for ROR2 expression (IHC score 2) were compared to those that stained weakly or not at all (IHC scores 1 and 0) (Fig. 1).

In DTF, we found no significant association between ROR2 expression and disease recurrence (HR = 0.9797, CI: 0.5268 to 1.822, P = 0.9482, Fig. S2). In GIST, tumors with high ROR2 expression were associated with decreased overall survival rates when compared to cases that expressed ROR2 weakly or not at all (HR = 1.417, CI: 1.060 to 1.893, P = 0.0186; Fig. 3A). In LMS, we found that patients whose tumor samples were strongly positive for ROR2 had a worse 5-year DSS than those whose tumor samples expressed ROR2 weakly or not at all. This reduction in DSS was seen in equal fashion in both GYN-LMS and ST-LMS (GYN-LMS: HR = 3.497, CI: 1.397 to 9.283, P = 0.0120; ST-LMS: HR = 3.287, CI: 1.234 to 8.756, P = 0.0173; Fig. 3B and 3C).

DISCUSSION

Experimental and clinical studies have shown that the deregulated RTKs can play important roles in cancer development and progression. Furthermore, RTKs have proven to be amenable therapeutic targets as is evidenced by several FDA-approved antibody and small molecule drugs targeting RTKs; these therapeutics have showed clinical efficacy in a wide range of cancer types.¹

Here we present RTK gene expression data in 148 soft-tissue sarcomas. One of the RTKs whose transcript levels was studied, ROR2, showed significant variability in expression in LMS, GIST and DTF. ROR2 is known to regulate cell migration during vertebrate development by acting as a receptor or co-receptor for Wnt5a.^{2,3} Recently, *in vitro* and xenograft experiments have shown that the Wnt5a-ROR2 signaling cascade is important for the invasive abilities of melanoma, osteosarcoma, and RCC cell lines, an effect that is likely mediated by ROR2 via Wnt5A-induced polarized cell migration, thereby making ROR2 a

candidate to explore as a biomarker of tumors with aggressive growth potential or as a novel therapeutic target.^{4-6,18}

In the current study, we present the first large-scale characterization of ROR2 expression in human soft-tissue sarcomas. The initial gene expression results were confirmed and expanded to a larger number of sarcomas on 573 cases representing 59 tumor types by IHC on TMAs. In addition to the tumor types identified by gene expression profiling, small numbers of ROR2-positive cases were found in high grade undifferentiated sarcoma and a significant subset of dermatofibrosarcoma protuberans. Similar to results shown for melanoma, osteosarcoma, and RCC cell lines, inhibition of *ROR2* expression strongly decreased the *in vitro* invasiveness of two ROR2-positive LMS and GIST cell lines. A third cell line, derived from an ROR2-negative LMS, exhibited no difference in invasion under the same experimental conditions, indicating that *ROR2* expression is functionally important for the subset of tumors in which it is present. Furthermore, treatment of these cells with ROR2-specific ligand Wnt5A increased cell invasion; upon ROR2 down-regulation, however, this increased invasion was significantly abrogated, thereby suggesting that in ROR2-positive LMS and GIST cells, the aggressive tumor phenotype induced by non-canonical Wnt signaling is likely mediated through ROR2. In all cell lines, reducing *ROR2* expression had no effect on cell viability or cell growth kinetics, suggesting that ROR2 specifically mediates the invasion and motility of these LMS and GIST cells.

The inhibitory effect seen on cell invasion suggests, but does not prove, a clinically significant role for the ROR2 molecule. To determine the possible role for ROR2 in the clinical behavior of tumors, we studied ROR2 expression on TMAs containing cases with known clinical follow-up. Here, we provide the first prognostic association of ROR2 protein expression with poor clinical outcome in cancer. In both LMS and GIST, ROR2 expression is significantly associated with poor clinical outcome.

Currently, there exist no targeted therapies for LMS, thereby making ROR2 an attractive therapeutic target given the prognostic associations and experimental findings reported herein. The majority of GIST tumors show activation of the tyrosine kinase proteins KIT or PDGFR α and specific mutations in the genes transcribing these proteins predict response to the tyrosine kinase inhibitor imatinib and other small molecule therapies. However, almost all GIST patients eventually develop resistance to treatment, thereby necessitating the exploration of other therapeutic targets, such as ROR2.^{15,16}

In summary, ROR2 is highly expressed in a subset of LMS, GIST, and DTF cases and high ROR2 protein expression is significantly associated with poor clinical outcome in patients with LMS and GIST. ROR2 expression is important for LMS and GIST cells to successfully migrate through a matrigel matrix, which implies an important role for ROR2 in mediating tumor invasive ability. Taken together, these results demonstrate the utility of ROR2 as a prognostic biomarker and suggest that ROR2 may represent a novel therapeutic target for the treatment of GIST, LMS, and possibly DTF.

SUPPLEMENTAL MATERIALS AND METHODS

Case material

For gene expression profiling, frozen tissue from 148 soft-tissue tumors was used; this included 61 GIST, 22 LMS, and 12 DTF (Table S2). For confirmation of ROR2 expression by IHC, we used a TMA with 573 cases from 59 sarcoma types (Table S1). For IHC studies on specimens with known clinical outcome data, we studied 410 GIST, 147 LMS, and 90 DTF, which were distributed over 10 TMAs (summarized in Table 1).^{9–12} These tumors were collected from Stanford University Medical Center, the University of Texas M.D. Anderson Cancer Center (UT-MDACC), and the Cancer Registry of Norway. All cases on the arrays consisted of material obtained at primary diagnosis and had accompanying follow-up data. Only four GIST cases had received imatinib therapy during the period of follow-up. The TMAs were constructed using 0.6mm cores with a manual tissue arrayer (Beecher Instruments, Silver Spring, MD, USA).

HEEBO gene arrays

The HEEBO microarray platform used in the study contained 44,544 70-mer probes that were designed using a transcriptome-based annotation of exonic structure for genomic loci. After confirmation of histology and the presence of viable tumor by frozen section, specimens were homogenized in Trizol reagent (Invitrogen, Carlsbad, CA, USA), and total RNA was extracted. The total RNA was reverse transcribed into cDNA using a mixture of oligo dT (Operon; high-performance liquid chromatography purified) and random hexamer (Amersham Biosciences, Little Chalfont Bucks, UK) primers with incorporation of amino allyl-dUTP (Ambion, Austin, TX, USA). Cy3 and Cy5 dyes (Amersham) were used for indirect labeling of the cDNA from reference RNA (Universal human reference RNA, Stratagene, La Jolla, CA, USA) and cDNA from tumor specimens, respectively. Microarray hybridization and washing was done using standard procedures.^{7,8} Microarrays were scanned on a GenePix 4000 microarray scanner, and fluorescence ratios (tumor/reference) were calculated using GenePix software. Only spots with a ratio of signal over a background of at least 1.3 in the Cy5 and 1.5 in the Cy3 channel were included. Gene centering was applied to the expression values for this series of tumors. Only genes with >50% available data were analyzed further by hierarchical clustering.

Cell culture

LMS04, LMS05, and GIST48 cells were derived from primary clinical specimens (LMS04: retroperitoneal lesion that spread from primary uterine LMS tumor; LMS05: primary thigh LMS tumor; GIST48: primary GIST with homozygous exon 11 KIT mutation (V560D) and heterozygous exon 17 KIT mutation (D820A)¹⁷). LMS04 and LMS05 cells were maintained in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 100 units/mL penicillin and streptomycin (Invitrogen) and 4mM L-glutamine (Invitrogen). GIST48 cells were maintained in IMDM (Invitrogen) supplemented with 15% FBS, 100 units/mL penicillin and streptomycin (Invitrogen) and 4mM L-glutamine (Invitrogen). All cell lines were cultured at 37°C in 5% CO₂ and the medium was replaced every 2 to 3 days.

Small interfering RNA transfections

LMS04 and LMS05 cells were seeded at densities of 8×10^4 cells per well (in 6-well plates) and 2×10^3 cells per well (in 96-well plates) in antibiotic-free medium and allowed to adhere overnight. Cells were transfected with a pool of control siRNAs (siNT, siGENOME Non-Targeting siRNA Pool #1, Dharmacon, Lafayette, CO, USA) or a pool of siRNAs targeting ROR2 (siROR2, ROR2 siGENOME SMARTpool, Dharmacon). Transfections were carried out with 20nM siRNA concentrations in OptiMEM (Invitrogen) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Efficiencies of siRNA knockdowns were assayed 24h, 48h, and 72h after transfection by quantitative real-time PCR. Cell growth kinetics and cell viability were quantified with a tetrazolium salt (WST-1) colorimetric assay (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's protocol.

Quantitative real-time PCR

RNA was extracted using Trizol (Invitrogen) using standard protocols. Gene expression was quantified using the SYBR green method. Primers were designed to cross intron-exon junctions: *ROR2* forward – GGCAGAACCCATCCTCGTG, reverse – CGACTGCGAATCCAGGACC; *Actin* forward – GCACCCAGCACAATGAAGA, reverse – CGATCCACACGGAGTACTTG. Quantitative real-time PCR was performed on a StepOnePlus instrument (Applied Biosystems, Foster City, CA). Transcript levels of target genes were analyzed using comparative C_t methods, where C_t is the cycle threshold number, and normalized to Actin.

Matrigel invasion assays

Invasion assays were performed using polyethylene terephthalate invasion chambers with 8.0µm pores (BD Bioscience, Bedford, MA, USA). Cells were transfected with siROR2 or siNT for 48h, serum-starved for 16h, counted, and seeded (2×10^5 cells) onto the filters. 20% FBS in RPMI was placed in the lower well to act as a chemoattractant. For Wnt5A treatment, 400ng/mL of recombinant human Wnt5A (R&D Systems, Minneapolis, MN) was added for 16h prior to invasion. Cells were allowed to invade for 24h before being fixed in 10% formalin, stained with crystal violet (Sigma-Aldrich, St. Louis, MO, USA), washed twice, and counted. All experiments were performed in triplicate.

ROR2 immunohistochemistry

Slides were cut to a thickness of 4µm, deparaffinized in xylene, and hydrated in a graded series of alcohol. The deparaffinized slides were then boiled by microwave for 12 minutes in citrate buffer (pH 6). A novel primary mouse anti-human ROR2 monoclonal antibody (generated by A.M. and R.N.) was used at a 1:25 dilution.¹³ The IHC reactions were visualized using mouse versions of the EnVision + system (DAKO, Carpinteria, CA, USA) using diaminobenzidine. Cores were scored as follows: 2: strong staining whether diffusely or focally present in the tumor; 1: weak staining whether diffusely or focally present in the tumor; 0: absence of any staining (Fig. 1). A score of 2 was considered positive for subsequent statistical analyses.

Statistical analyses

Kaplan-Meier analysis in GraphPad Prism V5.0 (GraphPad Software, San Diego, CA, USA) was used to generate survival curves with log-rank tests to compare patient outcome between groups. Student's t-test was used for comparison of the demographics data wherever appropriate. A p-value of less than 0.05 was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Representative immunohistochemical stains for ROR2 in LMS, GIST, and DTF
Samples were scored as follows: 2: strong staining whether diffusely or focally present in the tumor; 1: weak staining whether diffusely or focally present in the tumor; 0: absence of any staining (scale bar, 0.2mm). Examples of each score are shown for LMS, GIST, and DTF.

Figure 2. siRNA-mediated knockdown of ROR2 inhibits invasiveness of LMS and GIST cells
 ROR2 protein expression was analyzed by IHC of paraffin-embedded pellets of cell lines (left panels; scale bar, 35µm) and qRT-PCR (right panels) in LMS04, LMS05, and GIST48 (A). Treatment with siROR2 reduced transcript levels in LMS05 and GIST48 by nearly 70% as compared to siNT treatment (B). siROR2 treatment inhibits the invasion of ROR2-positive LMS05 and GIST48 cells through matrigel chambers, whereas no effect is seen in ROR2-negative LMS04 (C). Treatment of ROR2-positive LMS05 and GIST48 with ROR2-ligand Wnt5A increased cell invasion, an effect that was diminished by siROR2 treatment. The ROR2-negative LMS04 showed no response to treatment with Wnt5A (D). All experiments were performed in triplicate; error bars are $\pm$ one standard deviation.

Figure 3. ROR2 expression predicts poor clinical outcome in patients with GIST and LMS
Kaplan-Meier survival curves for GIST and LMS cases stratified by ROR2 protein expression. High ROR2 expression predicted poor overall survival in patients with GIST (A) and poor disease-specific survival in patients with both gynecological LMS (B) and non-gynecological LMS (C).

Table 1

ROR2 IHC scores for LMS, DTF, and GIST cases on TMAs with associated clinical outcome.

	Strong	Weak	Negative	Total
Leiomyosarcoma	47	67	33	147
Gastrointestinal stromal tumor	106	136	168	410
Desmoid-type fibromatosis	13	43	34	90