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Discoidin Domain Receptor Tyrosine Kinase 1 (DDR1) Is a Novel Therapeutic Target in Liposarcoma: A Tissue Microarray Study

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Abstract

Background Liposarcoma is the most commonly diagnosed subtype of soft tissue sarcoma. As these tumors often arise near vital organs and neurovascular structures, complete resection can be challenging; consequently, recurrence rates are high. Additionally, available chemotherapeutic agents have shown limited benefit and substantial toxicities. There is, therefore, a clear and unmet need for novel therapeutics for liposarcoma. Discoidin domain receptor tyrosine kinase 1 (DDR1) is involved in adhesion, proliferation, differentiation, migration, and metastasis in several cancers. However, the expression and clinical importance of DDR1 in liposarcoma are unknown.

Questions/purposes The purposes of this study were to assess (1) the expression, (2) the association between DDR1 and survival, and (3) the functional roles of DDR1 in liposarcoma.

Methods The correlation between DDR1 expression in tumor tissues and clinicopathological features and survival was assessed via immunohistochemical staining of a liposarcoma tissue microarray. It contained 53 samples from 42 patients with liposarcoma and 11 patients with lipoma. The association between DDR1 and survival in liposarcoma was analyzed by Kaplan-Meier plots and log-rank tests. The DDR1 knockout liposarcoma cell lines were generated by CRISPR-Cas9 technology. The DDR1-specific and highly selective DDR1 inhibitor 7RH was applied to determine the impact of DDR1 expression on liposarcoma cell growth and proliferation. In addition, the effect of DDR1 inhibition on liposarcoma growth was further accessed in a three-dimensional cell culture model to mimic DDR1 effects in vivo.

Results The results demonstrate elevated expression of DDR1 in all liposarcoma subtypes relative to benign

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lipomas. Specifically, high DDR1 expression was seen in 55% (23 of 42) of liposarcomas and no benign lipomas. However, DDR1 expression was not found to be associated with poor survival in patients with liposarcoma. DDR1 knockout or treatment of 7RH showed decreased liposarcoma cell growth and proliferation.

Conclusion DDR1 is aberrantly expressed in liposarcoma, and it contributes to several markers of oncogenesis in these tumors.

Clinical Relevance This work supports DDR1 as a promising therapeutic target in liposarcoma.

Introduction

Liposarcomas are a heterogeneous group of lipoblast-derived malignancies [28] that account for approximately 15% to 20% of all soft tissue sarcomas [13]. Of the four liposarcoma variants, the dedifferentiated and well-differentiated liposarcomas are especially challenging because they are more chemoresistant and more likely to arise within the retroperitoneum compared with the myxoid and pleomorphic types [28]. Although some argue that well-differentiated liposarcomas are benign, because they have no potential to metastasize, they do cause severe harm because of their propensity for recurrence and aggression within the retroperitoneum and mediastinum [26]. Dedifferentiated liposarcomas are deadly as well because they frequently are diagnosed late, after considerable growth [9]. Well-differentiated liposarcomas can transition into dedifferentiated liposarcomas, characterized by a heightened propensity for local recurrence and metastasis. Furthermore, for patients with advanced or metastatic soft tissue sarcomas such as liposarcoma, outcomes remain dismal with the median survival ranging from 12 to 15 months [12]. Complete and wide-margin surgical resection remains the only curative treatment for dedifferentiated and well-differentiated liposarcomas, and the greatest clinical success occurs when the tumor is localized to the extremity and can be resected with negative microscopic margins. However, aggressive surgical resection is important for retroperitoneal liposarcomas as well, even though it is rarely curative given that invasion into organs and vital neurovascular structures makes resection challenging [40]. At present, dedifferentiated liposarcomas within the retroperitoneum have recurrence rates approaching 60%, after which they form synchronous multifocal tumors only amenable to palliative care [4, 44]. The clear limitations of surgery have driven an expansion of studies investigating novel treatment strategies. Anthracycline-based chemotherapy combinations with ifosfamide or dacarbazine remain the standard first-line treatment for unresectable/metastatic liposarcoma, with gemcitabine plus docetaxel, trabectedin, and eribulin acting as subsequent options [17, 34, 41]. However, the benefit of these therapeutic regimens has been limited. It has been reported that chemotherapy is associated with a clinical benefit in 46% of patients with advanced well-differentiated liposarcomas and dedifferentiated liposarcomas, but overall survival remains poor. Current data highlight the urgent need for novel adjuvant therapies for liposarcomas.

Discoidin domain receptor 1 (DDR1) is a collagen-activated receptor tyrosine kinase with important roles in cancer cell adhesion, proliferation, differentiation, migration, and metastasis [5, 27]. Collagen is the most abundant component of the extracellular matrix and plays a crucial role in both cancer progression and chemoresistance via aberrant and complex signaling [11]. Liposarcoma is notable for its rich production of extracellular matrix components [9, 47]. We elected to examine DDR1 within liposarcoma as the retroperitoneal subtype is often housed within a collagenous stroma [43]. DDR1 has roles in the pathogenesis of various cancers such as melanoma, lung adenocarcinoma, breast cancer, colon cancer, pancreatic cancer, and gastric cancer, where its pharmacological

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inhibition blocks in vivo tumor growth [3, 24, 32, 35, 36, 49, 50]. During tumorigenesis, the activation and overexpression of DDR1 promotes tumor cell growth, proliferation, migration, and extracellular matrix remodeling; these result in cancer invasion and metastasis. Yet, despite DDR1's potential as an emerging therapeutic target, its expression and roles within liposarcoma are unknown. To our knowledge, no prior studies have examined DDR1 expression in liposarcoma patient specimens and cell lines as well as its functional roles in various markers of tumor progression.

After verifying its expression, we implemented the clustered regularly interspaced short palindromic repeats-associated protein-9 nuclease (CRISPR-Cas9) technology to generate DDR1 knockout liposarcoma cell lines, as it can precisely inactivate oncogenes for subsequent experimentation and precision therapy [19, 29]. CRISPR-Cas9 is a convenient and versatile platform for site-specific genome editing and epigenome-targeted modulation [6, 15, 29]. The cell lines included a dedifferentiated liposarcoma cell line SW 872 and a well-differentiated liposarcoma cell line 93T449 to encompass the subtypes known for retroperitoneal growth and chemoresistance [23, 28, 31]. We followed up by assessing the potent and selective DDR1 small molecule inhibitor 7RH to explore targeted DDR1 chemotherapy as a novel adjuvant treatment strategy for liposarcoma. The inhibitor 7RH has previously been shown to inhibit cell proliferation, invasion, adhesion, and tumorigenicity in cancer cells expressing high levels of DDR1 [18]. In other work, 7RH has shown preclinical success within in vitro and in vivo model systems of pancreatic cancer, breast cancer, gastric cancer, and nasopharyngeal carcinoma [1, 2, 18, 22, 30].

We therefore sought to assess (1) the expression, (2) the association between DDR1 and survival, and (3) functional roles of DDR1 in liposarcoma.

Materials and Methods

Liposarcoma Tissue Microarray and Immunohistochemistry

The human liposarcoma tissue microarray was purchased from Novus Biologicals LLC. The tissue microarray contained 53 samples from 42 patients with liposarcoma and 11 patients with lipoma; we had clinicopathological data on age, sex, tumor location, diagnosis, tumor tissue pathological subtypes, follow-up time and results, as well as the cause of death.

We determined the expression of DDR1 using immunohistochemical assays according to the manufacturer's instructions (Cell Signaling Technology). In brief, the paraffin-embedded slides were baked for 1 hour at 60°C before xylene deparaffinization and subsequent rehydration through graded ethanol (100% and 95%). A 3% hydrogen peroxide solution was used to quench endogenous peroxidase activity after heated epitope retrieval. After this, the slide was blocked for 1 hour with normal goat serum and then incubated with monoclonal rabbit antibody to human DDR1 (Cell Signaling Technology, 1:100 dilution in 1% bovine serum albumin phosphate-buffered saline [PBS]) overnight in a humidified chamber set at 4°C. SignalStain® Boost Detection Reagent (Cell Signaling Technology) and SignalStain® DAB (Cell Signaling Technology) were then used to detect the bound antibody. A hematoxylin QS (Vector Laboratories) counterstain was used to obtain clearer images of the liposarcoma cells before final long-term preservation using VectaMount AQ (Vector Laboratories) section mounting. Even in the absence of DDR1 antibody binding, the tissue microarray slides were stained to reveal any nonspecific secondary antibody reactions.

Analysis of IHC Staining in the Tissue Microarray

We used immunohistochemistry to determine the expression of DDR1. The tissue microarray slide was scored according to the percentage of DDR1 immunostaining, as assessed by two independent investigators (DCD, WF) who had no knowledge of the histopathological features or patient details of the samples. Any differences in the staining scores were resolved by consensus after joint review of the slides and discussion between the two investigators. Subsequently, we divided the DDR1 expression levels into four groups according to the intensity of cells showing positive staining: 0, no staining; 1+, weak staining; 2+, moderate staining; and 3+, strong staining. The low-DDR1 expression subset included groups 0 and 1+, whereas the high DDR1 expression subset included groups 2+ and 3+. Staining images were obtained using a Nikon microscope (Nikon Instruments Inc).

Cell Lines and Cell Culture

We purchased the wild type human liposarcoma cell lines from the American Type Culture Collection. All cell lines tested negative for mycoplasma and bacterial contamination. The SW 872 cell line is a dedifferentiated liposarcoma cell line derived from the connective tissue of a 36-year-old male, and the 93T449 cell line is a well-differentiated liposarcoma cell line derived from the retroperitoneum of a 68-year-old female (Supplementary Table 1; http://links.lww.com/CORR/B236). All liposarcoma cell lines were cultured at 37°C in a humidified 5% CO_2 atmosphere in RPMI 1640 (GE Healthcare Life Sciences) supplemented

with 10% fetal bovine serum (Sigma-Aldrich) and 1% penicillin/streptomycin (Thermo Fisher Scientific). The cells were resuspended with 0.05% trypsin-EDTA before subculture.

DDR1 CRISPR-Cas9 Knockout Design and Validation

CRISPR-Cas9 is a new technology for gene editing that is anticipated for treating genetic diseases such as cancer. The DDR1 knockout liposarcoma cell lines SW 872 and 93T449 were generated by CRISPR-Cas9 technology in a three-step process: guide design, editing optimization, and analysis of knockout efficiency with assistance by Synthego Corp [14, 39]. The single guide RNA (sgRNA) sequence was designed and selected based on maximal homology to the primary and alternative transcripts of DDR1, a high predicted on-target score, and a very low probability of off-target effects. The CRISPR components of sgRNA and Cas9 nuclease to form ribonucleoproteins were introduced into the liposarcoma cells by transfection. For genotyping, 48 hours posttransfection the genomic DNA was extracted by DNA QuickExtract (Lucigen), and the sgRNA-targeted and edited region was PCR-amplified. Sanger sequencing of the resulting PCR product was analyzed using Inference of CRISPR editing software to determine editing efficiency.

Protein Preparation and Western Blot

We used Western blot to confirm the decreased level of DDR1 in wild type and CRISPR-Cas9-edited liposarcoma cell lines SW 872 and 93T449. Total protein content was extracted from the wild type and knockout liposarcoma cell lines SW 872 and 93T449 using a mixture of 1× RIPA lysis buffer (Sigma-Aldrich) and protease inhibitor cocktail tablets (Roche Applied Science). Protein concentration was revealed with a determination reagent (Bio-Rad) and spectrophotometer (Molecular Devices Inc). Equal amounts of protein were separated in NuPAGE 4–12% Bis-Tris Gel (Thermo Fisher Scientific) and then transferred to a nitrocellulose membrane (Bio-Rad). After 1 hour of blocking with 5% nonfat milk, the membrane was incubated overnight with primary rabbit monoclonal antibodies to human DDR1 (D1G6 XP® Rabbit mAb, #5583, 1:1000 dilution, Cell Signaling Technology) at 4°C. After this incubation, Tris Buffered Saline Tween 20 (TBST) was used as a membrane wash (three times, 5 minutes, room temperature). Next, goat antirabbit secondary antibody IRDye 800CW (926-32211, 1:5000 dilution) (Li-COR Biosciences) was applied for 2 hours at room temperature followed by another TBST membrane wash (three times, 5 minutes, room temperature). Bands were detected using an Odyssey Infrared Fluorescent Western Blot Imaging System from Li-COR Bioscience, and Odyssey software 3.0 was used to quantify the bands.

Cell Growth and Proliferation Assays

Cell growth and proliferation assays were performed to determine the roles of DDR1 in liposarcoma cell growth and proliferation. Wild type and knockout liposarcoma SW 872 and 93T449 cells were seeded into 96-well plates at a density of 4×10^3 cells/well or six-well plates at a density of 6×10^5 cells/well for 5 days before quantification. After 5 days, the proliferation of the cells was assessed via MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay. After the growth period, 20 μL of MTT (5 mg/mL, Sigma-Aldrich) was added to each well of the 96-well plates. The cells were then incubated at 37°C in a humidified 5% CO_2 atmosphere for 4 hours, after which the resulting formazan product was solubilized with 100 μL of acid isopropanol, and the absorbance was measured at a wavelength of 490 nm using a SpectraMax Microplate® Spectrophotometer (Molecular Devices LLC). All MTT assays were performed in triplicate. Meanwhile, to detect the morphological changes of the cells, a Nikon microscope (Diagnostic Instruments Inc) was used after 5 days to detect changes in the six-well plates.

Immunofluorescence

We used an immunofluorescence assay to localize DDR1 in liposarcoma cells and validate the expression of DDR1 after CRISPR-Cas9 editing. Wild type and knockout liposarcoma SW 872 and 93T449 cell lines were grown in 12-well chambers for 60 to 65 hours. The samples were fixed with 4% paraformaldehyde (Sigma-Aldrich) in PBS for 15 minutes at room temperature. The samples were then permeabilized with ice-cold methanol (Sigma-Aldrich) for 10 minutes and then blocked in 1% BSA (Sigma-Aldrich) in PBST for 30 minutes to block unspecific binding of the antibodies. After incubation with rabbit DDR1 primary antibody (1:200 dilution, Cell Signaling Technology) or mouse monoclonal β-actin (1:200 dilution, Sigma-Aldrich) at 4°C overnight, samples were incubated with Alexa Fluor 488 (green) conjugated goat antirabbit antibody (A-11034, Invitrogen) and Alexa Fluor 594 (red) goat antimouse antibody (A-11032, Invitrogen) for 1 hour. Finally, Hoechst 33342 (1μg/mL, Invitrogen) was added to counterstain the cell nucleus. The cells were then imaged using a Nikon fluorescence microscope.

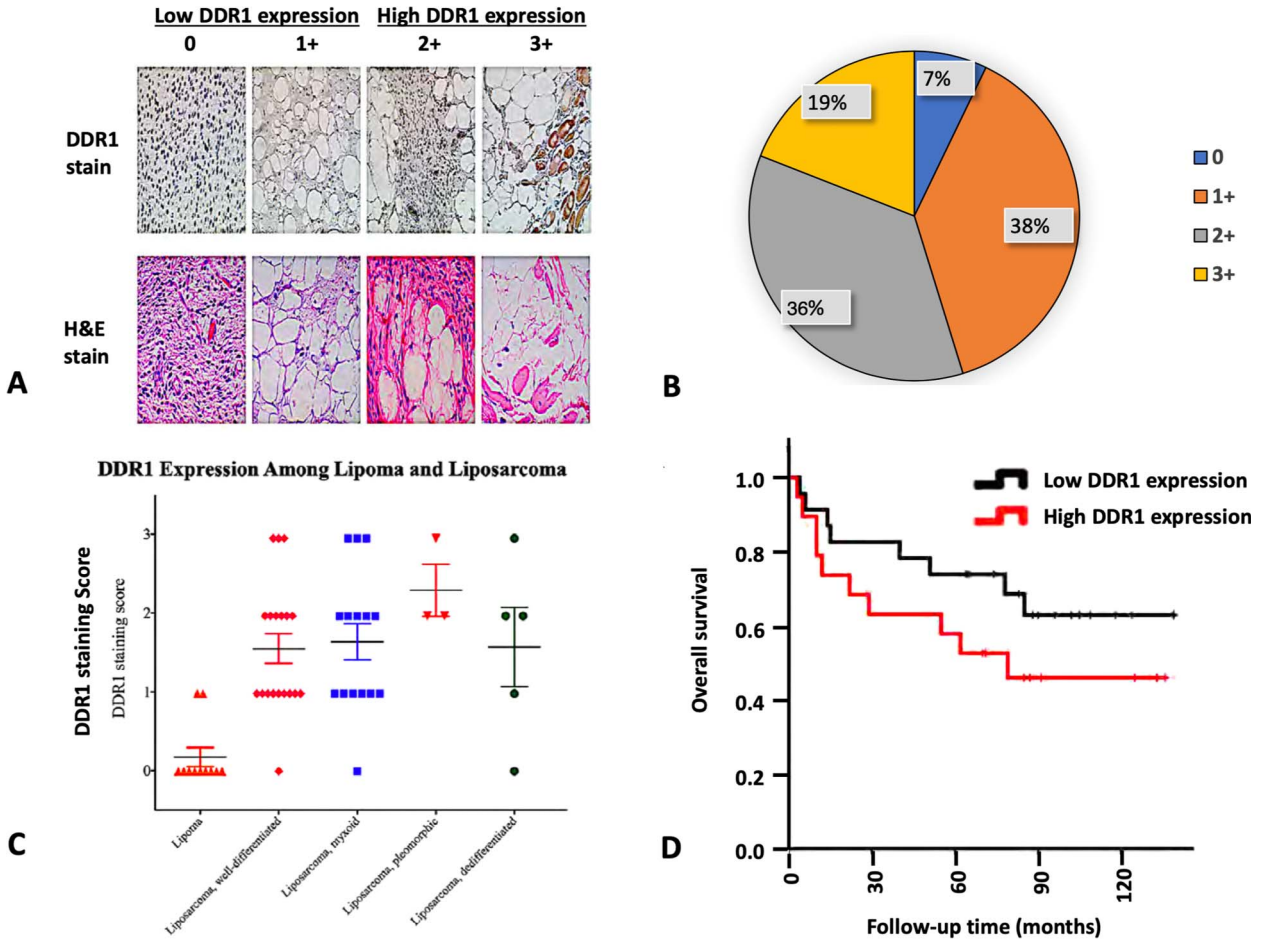

Fig. 1 DDR1 is overexpressed in liposarcoma and tends toward worse survival. (**A**) These are representative images of DDR1 staining along with hematoxylin and eosin staining in liposarcoma tissues. DDR1 expression levels were divided into four groups: 0, no staining; 1+, weak staining; 2+, moderate staining; and 3+, strong staining. The low DDR1 expression subset included groups 0 and 1+, whereas the high DDR1 expression subset included groups 2+ and 3+. (**B**) This pie chart represents relative frequency of different DDR1 expression levels in the liposarcoma tissue microarray. (**C**) This graphic represents DDR1 staining frequency between the lipomas and liposarcoma subtypes in the tissue microarray. (**D**) In this Kaplan-Meier overall survival curve, patients with liposarcoma were subgrouped as either the DDR1 low-expression group (staining score ≤ 1+) or the high-expression group (staining score ≥ 2+). Compared with the low-expression group, the patients with high DDR1 staining had a shorter overall survival but results did not reach clinical significance. A color image accompanies the online version of this article.

Cell Migration Activity

The effect of DDR1 on cell migration activity was evaluated with a wound-healing assay. In brief, 2×10^5 cells/well of wild type and knockout liposarcoma SW 872 and 93T449 cells were seeded onto 12-well plates. After the cells reached 100% confluence, they were wounded by scraping three parallel lines with a 200-μL tip, and then washed three times in serum-free medium and incubated in regular medium. Wounds were observed at 0, 24, 48, and 72 hours. Three images were taken per well at each time point using a Nikon microscope (10× objective) to monitor the cell repair process, and the distance between the two edges of the scratch (wound width) was measured at three random sites in each image. The cell migration distance was calculated by subtracting the wound width at each time point from the wound width at the 0-hour timepoint.

Three-Dimensional Cell Culture

To simulate the in vivo environment, a three-dimensional (3D) cell culture assay was used to evaluate the effect of DDR1 knockout on liposarcoma cell spheroid growth. Spheroids formed from the wild type and knockout liposarcoma cell lines SW 872 and 93T449 in 24-well VitroGel™ (The Well Bioscience Inc) 3D cell culture plates at a density of 2×10^5 cells/well were set up

Table 1. DDR1 expression in the lipomatous tumor tissue microarray

	DDR1 staining score			
Pathological subtype	**0**	**1+**	**2+**	**3+**
Lipoma	9 of 11	2 of 11	0	0
Liposarcoma	3 of 42	16 of 42	15 of 42	8 of 42
Well-differentiated	1 of 19	9 of 19	6 of 19	3 of 19
Myxoid	1 of 15	6 of 15	5 of 15	3 of 15
Pleomorphic	0	0	2 of 3	1 of 3
Dedifferentiated	1 of 5	1 of 5	2 of 5	1 of 5

The DDR1 staining score is summarized as follows: 39 of 42 showed positive DDR1 expression, ranging from staining 0 (3 of 42), 1+ staining (16 of 42), 2+ staining (15 of 42), and 3+ staining (8 of 42).

according to the manufacturer's protocol. The plates were incubated at 37°C in a humidified 5% CO_2 atmosphere. The medium was changed every 24 to 48 hours to provide enough nutrients for cell growth and to prevent an osmolality shift of the medium. The spheroids were photographed every 2 days with a Nikon microscope. At the 12-day point, the spheroids were harvested from the bottom of the plate by gentle pipetting of 100 μL of PBS into each well. After 15 minutes of incubation with 0.25 μM Calcein AM (Invitrogen), the spheroids were imaged using a Nikon fluorescence microscope.

DDR1 Expression and Survival

The patient samples were representative of the typical adult liposarcoma patient, as the median (range) age of liposarcoma patients included in the tissue microarray was 53 years (28 to 88). As a reference, in the US Surveillance, Epidemiology, and End Results (SEER) public-access database, the median age of liposarcoma diagnosis between 1973 and 2006 was 60 years [16]. The median (range) follow-up time of patients in this study was 88 months (3 to 145).

Table 2. Clinicopathological features of patients whose tumors were used in lipomatous tumor tissue microarray

		DDR1 expression		
Clinicopathological features	**Number of patients**	**Low**	**High**	**p value**
All patients	53	30 of 53	23 of 53	
Age in years				0.50
< 60	39 of 53	21 of 39	18 of 39	
≥ 60	14 of 53	9 of 14	5 of 14	
Sex				0.82
Male	24 of 53	14 of 24	10 of 24	
Female	29 of 53	16 of 29	13 of 29	
Location				0.80
Head and neck	4 of 53	3 of 4	1 of 4	
Mediastinum	1 of 53	1 of 1	0	
Intraabdomen	10 of 53	4 of 10	6 of 10	
Retroperitoneum	11 of 53	6 of 11	5 of 11	
Trunk	7 of 53	5 of 7	2 of 7	
Upper extremity	9 of 53	5 of 9	4 of 9	
Lower extremity	11 of 53	6 of 11	5 of 11	
Pathological subtype				0.009
Lipoma	11 of 53	11 of 11	0	
Liposarcoma, well-differentiated	19 of 53	10 of 19	9 of 19	
Liposarcoma, myxoid	15 of 53	7 of 15	8 of 15	
Liposarcoma, pleomorphic	3 of 53	0	3 of 3	
Liposarcoma, dedifferentiated	5 of 53	2 of 5	3 of 5	
Disease status				0.65
Alive	35 of 53	20 of 35	15 of 35	
Dead from disease	17 of 53	9 of 17	8 of 17	
Dead from another cause	1 of 53	1 of 1	0	

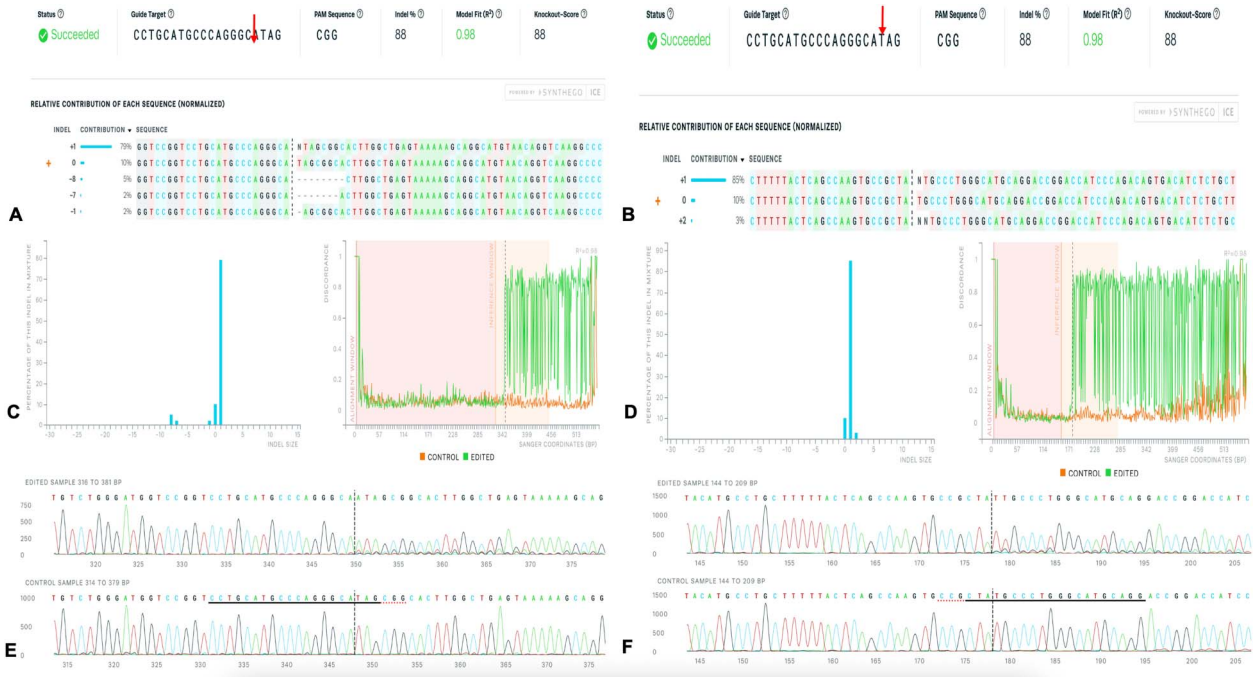

Fig. 2 Analyses of CRISPR-Cas9 knockout DDR1 liposarcoma cell lines SW 872 and 93T449. (**A-B**) The contributions show the inferred sequences present in the edited population and their relative proportions in liposarcoma cell lines (**A**) SW 872 and (**B**) 93T449. Cut sites are represented by a red arrow in the guide target sequences, a black vertical dotted line in edited sequences, and a wild-type sequence is marked by a red "+" symbol on the left. (**C-D**) Indel distributions of CRISPR-Cas9 edited liposarcoma cell lines (**C**) SW 872 and (**D**) 93T449. The Indel plots, shown on the left in C-D, display the inferred distribution of indels in the entire edited population of genomes. Hovering over each bar of the Indel plot shows the size of the insertion or deletion (+ or - 1 or more nucleotides), along with the percentage of genomes that contain it. The discordance plots, shown on the right in C-D, detail the level of alignment per base between the wild type (control) and the edited sample in the inference window (the region around the cut site), that is, it shows the average amount of signal that disagrees with the reference sequence derived from the control trace file. On the plot, the green line and orange line should be close together before the cut site, with a typical CRISPR edit resulting in a jump in the discordance near the cut site and continuing to remain far apart after the cut site (representing a high level of sequence discordance). (**E-F**) The Sanger sequence view showing edited and wild type (control) sequences in the region around the guide sequence of liposarcoma cell lines (**E**) SW 872 and (**F**) 93T449.

Primary and Secondary Study Outcomes

Our primary study goal was to determine the expression of DDR1 in liposarcoma tissue microarray by immunohistochemistry. The association between DDR1 expression and survival of liposarcoma patients was analyzed by Kaplan-Meier plots and log-rank tests.

The secondary goal of our study was to define the functional roles of DDR1 in liposarcoma cell growth and proliferation. For this purpose, we used CRISPR-Cas9 technology to knockout the DDR1 liposarcoma cell lines. The effects of DDR1 on liposarcoma cell growth and proliferation were further validated by DDR1 inhibitor 7RH in both two-dimensional (2D) and 3D cell culture models.

Ethical Approval

Ethical approval was not sought for the present study. The human liposarcoma tissue microarray used in this study was purchased from Novus Biologicals LLC and used in accordance with the policies of the Ethics Committee of Sylvester Comprehensive Cancer Center and the University of Miami Miller School of Medicine, and all methods were carried out in accordance with relevant guidelines and regulations.

Statistical Analysis

We used GraphPad Prism version 8.0 software for statistical analyses. Independent two-tailed Student t-tests were performed to analyze the differences between two groups. Differences in survival were analyzed by Kaplan-Meier plots and log-rank tests. The relationship between DDR1 expression and liposarcoma patient clinicopathological features were evaluated by the $\chi 2$ test, and p values less than 0.05 were considered statistically significant. Results are presented as the average of experiments performed in triplicate.

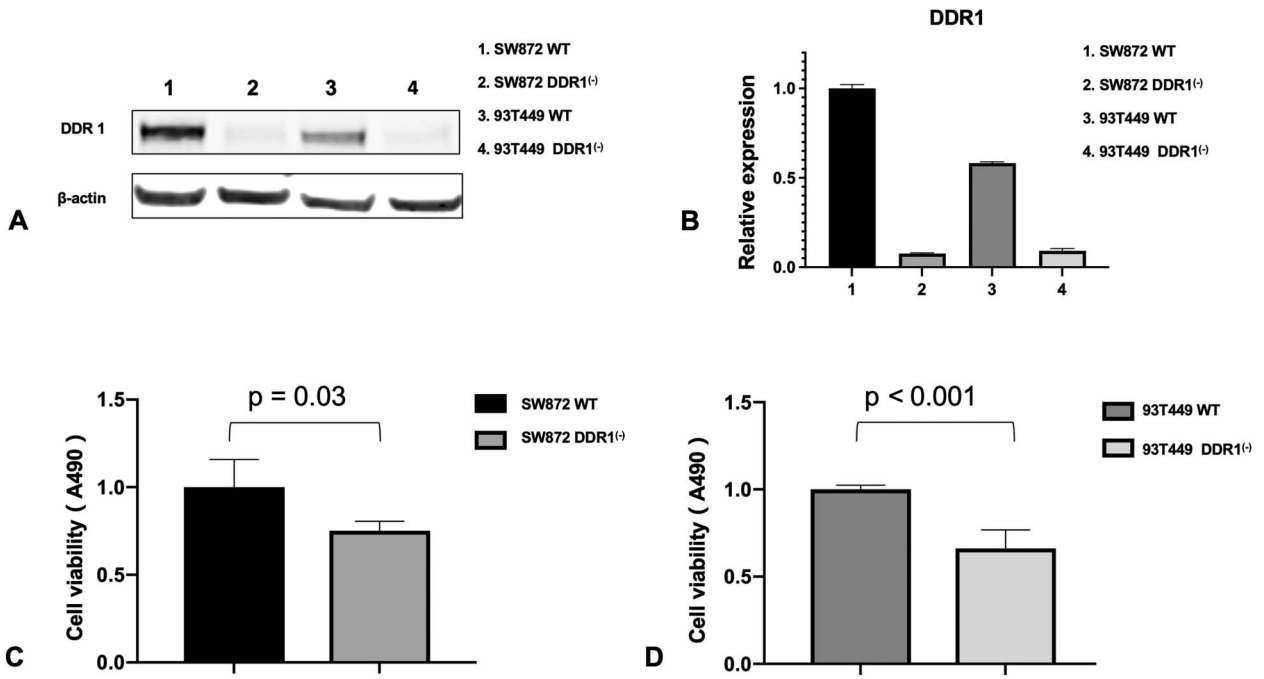

Fig. 3 DDR1 CRISPR-Cas9 knockout decreased liposarcoma cell viability. (**A**) Additional confirmation of DDR1 gene knockout in SW 872 and 93T449 cell lines by Western blot assays and (**B**) their relative expression. Liposarcoma cell proliferation assays in (**C**) 93T449 and (**D**) SW 872 cell lines, which were determined by MTT assays after 5 days of growth.

Results

DDR1 Expression

We first elected to evaluate DDR1 expression in liposarcoma patient tissue samples to verify whether it was, in fact, a potential therapeutic target. As shown by the liposarcoma immunohistochemical staining, DDR1 was most expressed in the liposarcoma tissues compared with the benign lipomas (Fig. 1A-C). Of the 42 liposarcoma patient tissues in the tissue microarray, 93% (39 of 42) showed positive DDR1 expression, ranging from staining group 0 (3 of 42), 1+ staining (16 of 42), 2+ staining (15 of 42), and 3+ staining (8 of 42) (Table 1). The stained liposarcoma specimens were then subdivided into two categories: 0 and 1+ were defined as the low DDR1 expression group (45% [19 of 42]), whereas the 2+ and 3+ staining groups were defined as the high DDR1 expression group (55% [23 of 42]). In contrast, of the 11 lipoma patient tissues, expression of DDR1 was limited to the low expression staining group including 0 (9 of 11) and 1+ (2 of 11) (Table 1). Relative to lipoma, all liposarcoma subtypes including well-differentiated, myxoid, pleomorphic, and dedifferentiated showed greater DDR1 expression, with pleomorphic liposarcoma having the highest expression (Fig. 1C).

Association Between DDR1 Expression and Survival

Next, we evaluated the association between DDR1 expression in the tissue microarray and liposarcoma patient survival (Table 2). Despite increased expression of DDR1 in liposarcomas compared with lipomas, DDR1 expression was not associated with inferior survival time in patients with liposarcomas (103 months [95% CI 81 to 124] in patients with low DDR1 expression versus 84 months [95% CI 58 to 109] in those with high DDR1expression; p = 0.33) (Fig. 1D).

Further analysis showed DDR1 expression was unrelated to other patient clinicopathological features including age, sex, and tumor site (Table 2).

Functional Roles of DDR1 in Liposarcoma

DDR1 CRISPR-Cas9 Knockout Design and Validation

We next sought to determine the presence and expression of DDR1 in human liposarcoma cell lines. Western blot testing revealed that DDR1 was strongly expressed in both dedifferentiated (SW 872) and well-differentiated (93T449) liposarcoma cell lines. We then elected to use CRISPR-Cas9 technology to produce their respective DDR1 knockout

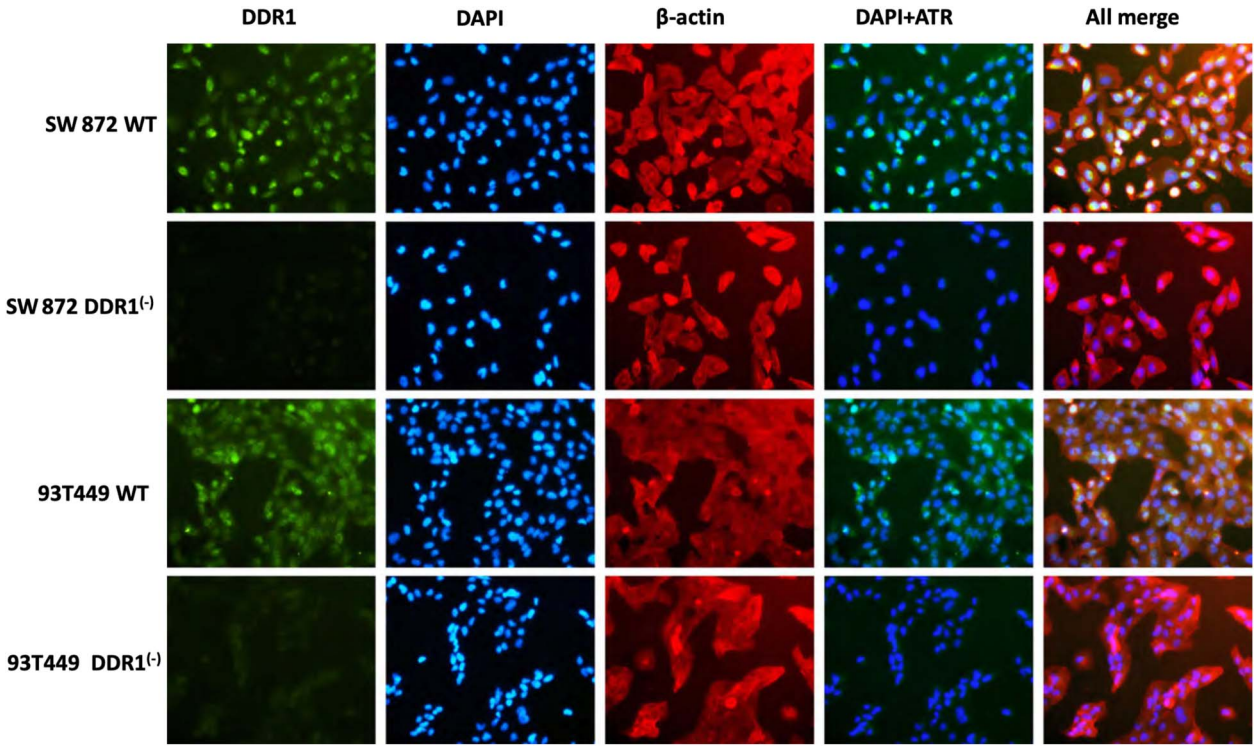

Fig. 4 DDR1 knockout induced cell death, as shown by immunofluorescence. DDR1 expression in liposarcoma cell lines, with groups including wild type and knockout SW 872 and 93T449 cells. Immunofluorescence signals include DDR1 (green), β-actin (red in cytoplasm) and DAPI (4′,6-diamidino-2-phenylindole) that binds strongly to adenine-thymine rich regions in DNA (blue in nuclei), and ATR (yellow in cytoplasm). The green fluorescence signal indicates that the DDR1 protein was primarily localized to the nucleus of active liposarcoma cells with expression extending to the cell membrane and cytoplasm and was clearly inhibited and showed decreased viability following CRISPR-Cas9 knockout.

counterparts, as it affords a high-resolution view of cancer cell vulnerabilities and targets [20]. The guide target sequence used was CCTGCATGCCCAGGGCA*TAG, with the cut site occurring at the asterisk and a PAM sequence of CGG. After DNA extraction, Sanger sequencing of the resulting PCR product showed an 88% indel rate and knockout score via Inference of CRISPR editing software (Fig. 2). Additionally, a model fit (R^2) of 0.97 was achieved, which is a measure of how well the proposed indel distribution fits the Sanger sequence data of the edited sample (Fig. 2). We performed confirmatory Western blots in the knockout cell lines, and as expected, found higher DDR1 expression within the wild type cell lines relative to the knockouts (Fig. 3A-B). Overall, we demonstrated that DDR1 is expressed in liposarcoma cell lines and can be successfully targeted and knocked out within the genome by CRISPR-Cas9.

DDR1 Knockout Suppresses Liposarcoma Cell Viability and Proliferation

To validate the role of DDR1 in cell proliferation and growth, we implemented an MTT assay, which is a colorimetric assay that reflects the number of viable cells present by assessing cell metabolic activity [8]. Over a 5-day culture period, we observed decreased cell viability in the DDR1 knockouts of both cell lines relative to their wild type counterparts (SW 872 WT versus SW 872 DDR1(-); $p = 0.03$ and 93T449 WT versus 93T449 DDR1(-); $p < 0.001$, Student two-tailed t-test) (Fig. 3C-D).

DDR1 Knockdown Induces Cell Death and Decreases DDR1 Expression as Confirmed by Immunofluorescence

As an additional confirmatory step, the effects of DDR1 activation and expression on liposarcoma cell growth and the subcellular localization of DDR1 were evaluated by an immunofluorescence assay. The assay showed that in addition to the cell membrane, the DDR1 protein also localized within the nucleus of activated liposarcoma cells a mechanism that has been described previously in tissue turnover and fibrosis [10]. The DDR1 immunofluorescence analysis further confirmed a reduction of cell viability in both knockout versions of the SW 872 and 93T449 cell lines relative to their wild type cells. As expected, those

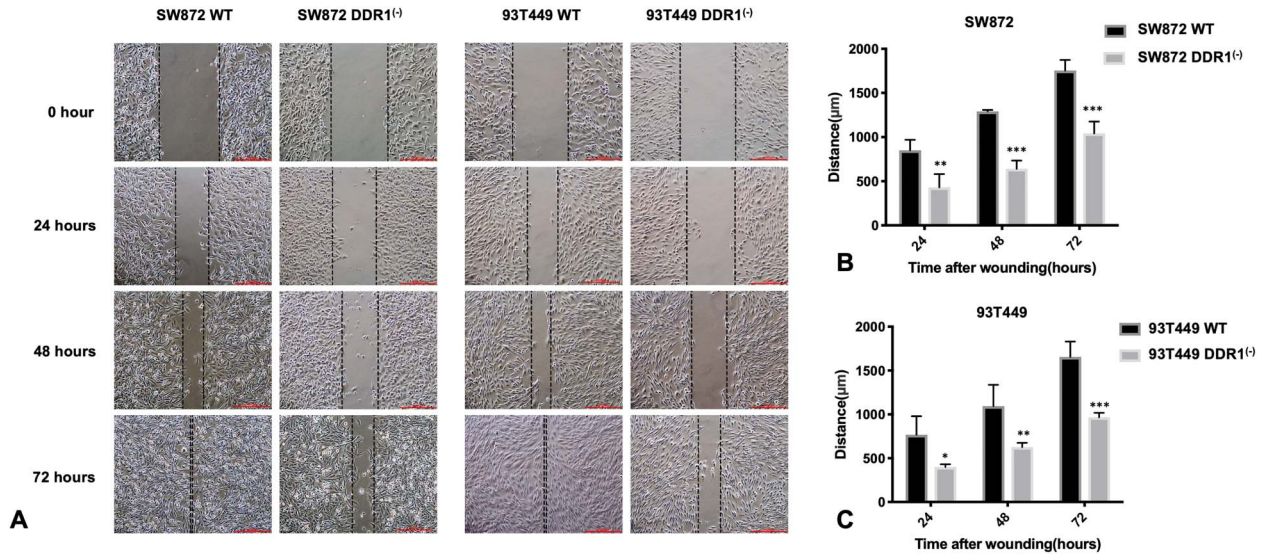

Fig. 5 DDR1 CRISPR-Cas9 knockout decreased liposarcoma cell migration. (**A**) The SW 872 and 93T449 cell line migration with and without DDR1 knockout. Distances were measured after 24 hours, 48 hours, and 72 hours with quantification of (**B**) SW 872 and (**C**) 93T449 demonstrating statistical significance; p value: < 0.033 (*), < 0.002 (**), < 0.001 (***).

liposarcoma cells that underwent DDR1 knockout expressed much lower levels of DDR1 (Fig. 4).

Knockout of DDR1 Reduces Liposarcoma Cell Motility and Spheroid Growth

The wound healing assay is a standard in vitro technique for analyzing the migration that commonly occurs in metastasis [25]. We performed this assay in the DDR1 knockout and wild type cell lines, with relative cell migration distance evaluated at 0, 24, 48, and 72 hours. After treatment with the wound healing assay as described, we observed a marked inhibition of migratory potential in both SW 872 and 93T449 DDR1 knockout cell lines compared with wild type cells ($p < 0.05$). Wounds were almost fully recovered after the 72-hour migration period in the wild type cells. These data demonstrate that inhibition of DDR1 impairs liposarcoma cell motility and possible metastatic migration (Fig. 5).

Because flat 2D culture systems may not adequately mimic the in vivo conditions by which liposarcoma cells attach, spread, and grow in 3D, we evaluated how DDR1 alters liposarcoma growth within a simulated in vivo 3D culture environment. Observations of spheroid size were recorded across several timepoints, and although the spheroids continuously grew, the spheroid diameters in DDR1 knockout SW 872 and 93T449 cells were smaller than the untreated cells ($p < 0.001$ two-way ANOVA). Collectively, our results further support that DDR1 has a crucial role in liposarcoma growth and progression in vitro (Fig. 6).

DDR1 Inhibitor 7RH Suppresses Liposarcoma Cell Viability and Proliferation

To further characterize the functional role of DDR1 in liposarcoma, we evaluated the effect of DDR1 inhibition by a potent and selective DDR1 inhibitor on liposarcoma cell lines. Specifically, we treated SW 872 and 93T449 with increasing concentrations (0.00002-10 μM) of 7RH over 5 days and subsequently examined cell viability morphologically under microscopy and via MTT assay. Over a 5-day culture period with increasing 7RH concentrations, we observed morphological changes such as cell shrinking and decreased cell viability in both cell lines (Fig. 7).

Discussion

Although previous studies have shown heightened DDR1 expression correlates with pathological classification, clinical characteristics, treatment response, and worse survival in several cancers [21, 22, 38, 46], there are no studies to our knowledge detailing its expression and importance in liposarcoma. Within patient tissues, DDR1 was present in 93 of the liposarcomas, highly expressed in 55% of the liposarcomas, and they did not have high expression in any of the benign lipomas. We found that DDR1 expression was not associated with reduced survival, although this could be due to the insufficient sample size, and therefore should be further explored in future studies comparing tumor specimens from a large cohort of liposarcoma patients.

Limitations

In our study, the sample size of clinical cases is relatively small, which limits our ability to detect differences at subgroup levels. Additionally, the exact molecular mechanism underlying the impact of DDR1 knockout on the growth and migration of liposarcoma cells requires further investigation. Although 3D cell culture can mimic in vivo conditions, the lack of in vivo animal experiments is a limitation of our research. Further studies are needed to address these gaps and provide a more comprehensive understanding of the role of DDR1 in liposarcoma.

Discussion of Key Findings

The liposarcoma tissue microarray demonstrated that DDR1 was principally localized to the cell membrane and highly expressed in both tested human liposarcoma cell lines. We did, however, also observe that DDR1 was expressed within the nucleus of highly proliferative liposarcoma cells in our immunofluorescence assay. Although principally considered a membrane-bound receptor, this finding is consistent with previous works in injured kidneys, whereby DDR1 translocated to the nucleus to produce profibrotic molecules [10]. A recent study also showed DRR1 can be localized in the membrane, cytoplasm, and nuclear compartments of both normal and cancerous prostate epithelial cells [7].

As pharmacological inhibition of DDR1 has shown to reduce tumor burden and chemoresistance in several cancers [1-3], we analyzed whether similar anticancer effects occurred in liposarcoma. We used a dedifferentiated (SW 872) and well-differentiated (93T449) liposarcoma cell line for our investigation. Therapeutically, we employed CRISPR-Cas9-based genome engineering, as it can precisely target an oncogene for experimentation or therapeutics and can be verified by detailed Sanger sequencing analysis [37, 48]. The expression of DDR1 in both human liposarcoma cell lines was successfully knocked out by CRISPR-Cas9, resulting in decreased proliferation and migration. Additionally, because 3D cell models more accurately translate in vitro results for in vivo application than traditional 2D plating techniques [33, 45], we further validated the effect of DDR1 knockout on cell proliferation using 3D cell cultures to better mimic the tumor microenvironment. We observed a decrease of cell spheroid diameter in the DDR1 knockout cell lines.

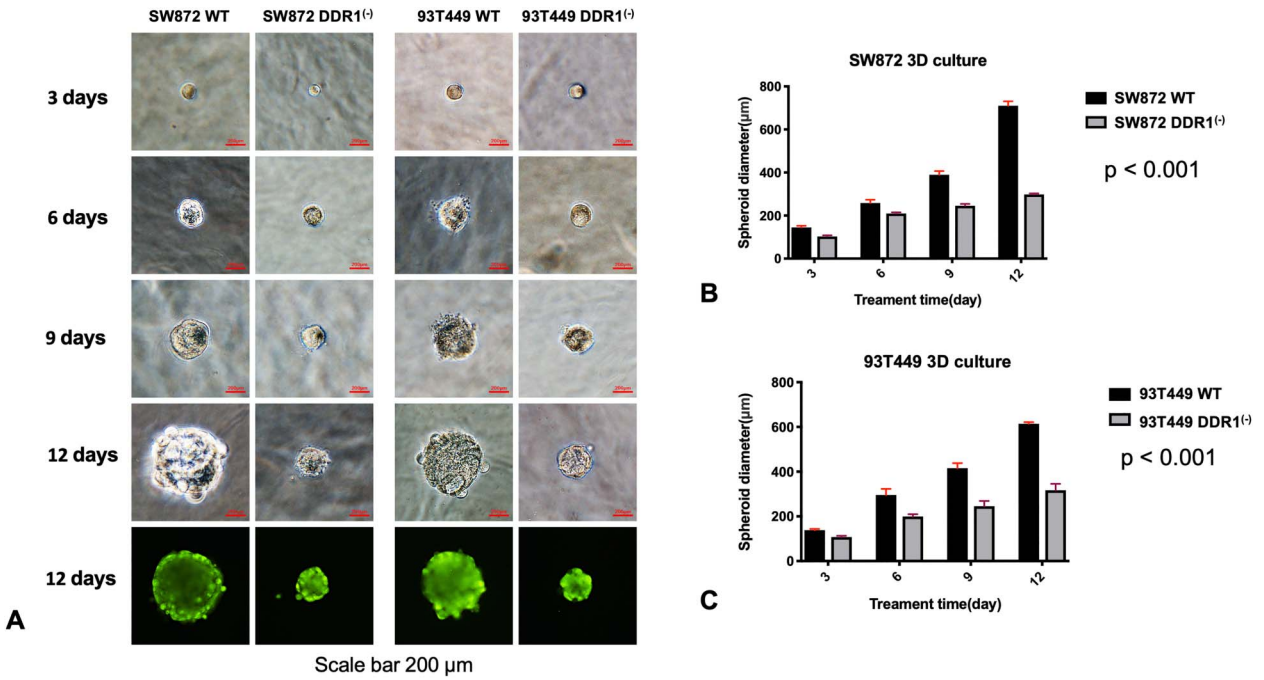

Fig. 6 DDR1 CRISPR-Cas9 knockout decreased liposarcoma cell spheroid formation. (**A**) Representative images of SW 872 and 93T449 cell lines in 3D culture with and without DDR1 knockout. Cell fluorescence images of spheroid formation were taken after 12 days of cultivation. (**B**) SW 872 and (**C**) 93T449 knockouts were smaller than wild type cells at all observation points. A color image accompanies the online version of this article.

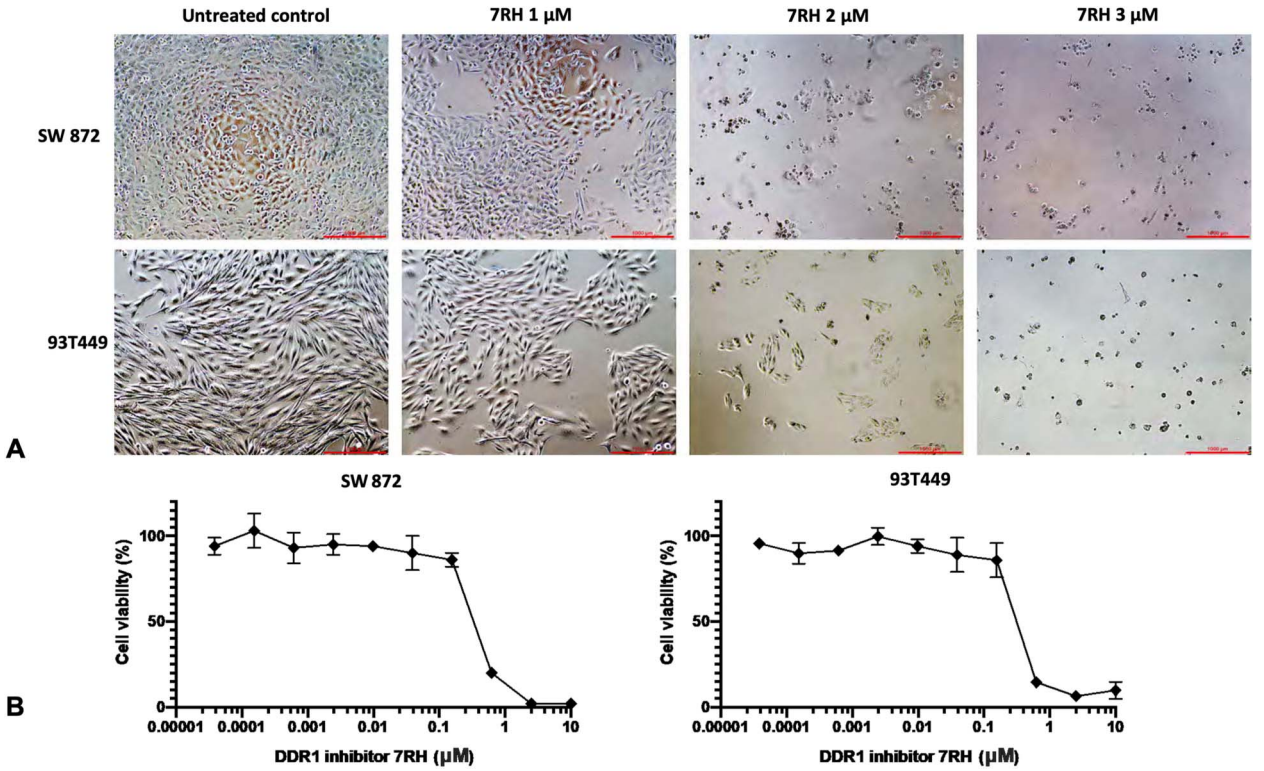

Fig. 7 DDR1 inhibition by 7RH decreased liposarcoma cell proliferation. (**A**) Representative microscopy images of SW 872 and 93T449 cell lines after 5 days, with and without 7RH treatment at indicated concentrations. (**B**) The inhibition of liposarcoma cell proliferation of SW 872 and 93T449 was determined by MTT assays.

Finally, as the selective and potent DDR1 inhibitor 7RH has shown preclinical success in pancreatic ductal adenocarcinoma [1], gastric carcinoma [22], and nasopharyngeal carcinoma [30], we analyzed its treatment potential in our liposarcoma cell lines. Results of a previous in vitro study in which DDR1 was inhibited in cancer cell lines with elevated expression have shown it to be an effective means for decreasing proliferation, invasion, adhesion, and tumorigenicity [18]. 7RH has been shown to block collagen-induced DDR1 signaling in pancreatic tumor cells and consequently reduce colony formation and migration [1]. Inhibition of DDR1 with 7RH has also shown efficacy in combination with other chemotherapeutics or immunotoxin therapy in several cancer cell lines and tumor-orthotopic xenografts, where it reduced DDR1 activation and downstream signaling, primary tumor burden, and chemoresistance [1, 2, 18]. A more recent study showed DDR1 initiates immune exclusion by promoting collagen fiber alignment. CRISPR knockout DDR1 in tumors promotes the intratumoral penetration of T cells and obliterates tumor growth in mouse models of triple-negative breast cancer [42]. Consistent with these observations, in this study, we observed 7RH to reduce liposarcoma cell growth and proliferation in a dose-dependent manner. Therefore, our results are consistent with those in previously studied cancers and indicate DDR1 also plays important roles in the growth and proliferation of liposarcoma cells.

Conclusion

We confirmed the successful knockout of DDR1 via genome editing with CRISPR-Cas9 and inhibition with a selective and potent DDR1 small-molecule inhibitor 7RH. Decreased DDR1 expression reduced several established parameters of tumor progression, including growth, proliferation, migration, and spheroid formation. Overall, our targeted genetic analysis of DDR1 in liposarcoma is promising, and it warrants future investigation with larger sample sizes, which may better capture its correlation with patient outcomes.

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