The oncogene LRF is a survival factor in chondrosarcoma and contributes to tumor malignancy and drug resistance

Introduction

Chondrosarcoma is the second most common primary tumor of bone (1). The treatment of chondrosarcoma is usually limited to wide-margin surgical resection because it is highly resistant to conventional chemo- and radiotherapy, and hence prognosis is poor for unresectable and metastatic diseases (2,3). The benign form of the tumor is known as chondroma. Correctly distinguishing between the two forms is essential for making therapeutic decisions. However, due to their similar histological appearances and the lack of a reliable diagnostic marker, it is often difficult to distinguish benign tumors from low-grade chondrosarcoma. Therefore, it is necessary to search for a potential marker that has diagnostic and prognostic values in chondrosarcoma. In this study, we demonstrated by immunohistochemistry that elevated leukemia/lymphoma-related factor (LRF) expression was associated with increased malignancy in human chondrosarcoma tissue microarrays. Moreover, siRNA depletion of LRF drastically reduced proliferation of chondrosarcoma cell lines and effectively induced senescence in these cells. This could be attributed to the observation that LRF-depleted cells were arrested at the G₁ phase, and had increased p53 and p21 expression. Moreover, LRF depletion not only drastically reduces the cellular migration and invasion potentials of chondrosarcoma cells but also sensitized these cells to the apoptosis-inducing chemotherapeutic agent doxorubicin. We conclude that LRF is a survival factor in chondrosarcomas and its expression correlates with tumor malignancy and chemoresistance. Our data implicate the potential role of LRF as both a diagnostic marker and therapeutic target for chondrosarcomas.

Materials and methods

Cell lines and reagents

The two human grade II chondrosarcoma cell lines: FS090 was a kind gift from Dr Joel A. Block (Rush University) and SW1353 was purchased from ATCC. Cells were cultured in Dulbecco’s Modified Eagle’s Medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), penicillin (100 U/ml) and streptomycin (100 µg/ml) (Invitrogen) at 37°C with 5% CO₂. Doxorubicin (Sigma) was dissolved in sterile water to make a stock solution of 10mM. Polyclonal antibodies against LRF (catalog no. SC-66953), p53 (SC-126), p21 (SC-397) and horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology. Monoclonal antibody against glyceraldehyde 3-phosphate dehydrogenase was from Ambion.

Chondrosarcoma tissue microarrays and immunohistochemical staining of LRF

Human chondrosarcoma tissue microarrays were purchased from Cybrid (CS36-01-001) and US Biomax (B02081). LRF staining was performed with a polyclonal rabbit anti-LRF antibody used previously in the studies of other human cancers (10,11). Briefly, antigen retrieval was achieved with a commercial antigen retrieval solution (CS36-01-001) and US Biomax (B02081). LRF staining was evaluated by two blinded observers and the interobserver variability was <5%. The semiquantitative scoring scheme used for evaluation of intensity of nuclear staining was as follows: 0 = undetectable, 1+ = weakly positive, 2+ = moderately positive and 3+ = strongly positive. The percentage of LRF-positive cells from each sample was determined by counting cells at 10x magnification in three different optical fields.

Lentiviral constructs

The two LRF-targeting small interfering RNAs (siRNAs) used in this study were GCTGCTGCAAGACTGATGTG (sense) and ATGGACTACTACCTGAAATAC (antisense). A siRNA targeting enhanced green fluorescent protein (EGFP) was used as control. All siRNA sequences were inserted into the AgeI and EcoRI sites of the lentiviral vector pLKO.1 (plasmid no. 8453, www.Addgene.com). Lentiviral particles were generated and titered as described previously (13). FS090 and SW1353 cells were seeded in 6-well plate at 70–80% confluency and grown for 24 hours. Doxorubicin treatment was started 24 hours after lentiviral transduction.
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24h prior to transduction. Lentiviral particles harboring LRF- or EGFP-siRNAs or EGFP-siRNA were then added at 10 multiplicity of infection in the presence of 1 µg/ml polybrene. The medium was replaced after 16h and the cells were used for various experiments after 6–12 days. 

Proliferation and cytotoxicity assays
For the proliferation assay, 1 × 10⁴ cells were seeded in 96-well plates and the mitochondrial dehydrogenase activity was measured every 2 days for up to 4 days, using a water-soluble tetrazolium (WST)-based proliferation assay kit (Biovision) following the manufacturer’s protocol. For cytotoxicity assay, 7 × 10⁴ cells/well were seeded in 96-well plates, followed by doxorubicin treatment at various concentrations (0.03, 0.13, 0.67 µM for FS090 and 0.02, 0.08, 0.39 µM for SW1353 cell line) for 24h. The next day, the medium containing doxorubicin was removed and fresh medium was added and cells were grown for an additional 24h before performing the WST assay.

Clonogenic assay
To determine the abilities of cells to form colonies, 1 × 10⁴ cells expressing LRF- or EGFP-siRNA were seeded into 6-well plates and allowed to grow for 3 weeks, with media changes every 3–4 days. The colonies were stained with 0.05% crystal violet (Sigma) for 1h at room temperature, washed two times with phosphate-buffered saline (PBS), and visualized under the microscope.

Cell cycle analysis
Cell cycle analysis was performed by flow cytometry after propidium iodide staining. Cells were transduced with lentivirus harboring LRF- or EGFP-targeting siRNA and allowed to grow for 8 days. Cells were harvested by trypsinization and washed with PBS, followed by fixation in 70% ethanol for 30 min on ice. Cells were then washed, resuspended in PBS containing 200 µg/ml RNase (Fermentas) and incubated at 37°C for 30 min. Propidium iodide (Roche) was added to a final concentration of 25 µg/ml and cells were incubated on ice for 30 min. Propidium iodide staining (a minimum of 10,000 events were counted for each sample) was detected by flow cytometry (FACS Fortessa LSR, Becton Dickinson) and the cell cycle profiles were analyzed by Flowjo software.

β-Galactosidase and LRF double staining
Cellular senescence was detected by a senescence detection kit (Biovision), following the manufacturer’s protocol. For each sample, 5 × 10⁴ cells were seeded in multi-chamber slides and allowed to adhere overnight. Cells were fixed for 10min at room temperature, washed with PBS, and β-galactosidase substrate solution was added. Cells were incubated at 37°C in 5% CO₂ for 18h and then washed twice with PBS. Cells were examined using light microscopy at 10x magnification (Nikon, ECLIPSE TE 2000), and the percentage of senescent cells was determined by counting cells in three different optical fields. For double staining with anti-LRF antibody, β-galactosidase staining was performed as described above, the cells were then permeabilized in 0.1% Triton X-100 in PBS for 10min on ice, followed by blocking in PBS containing 5% bovine serum albumin at room temperature for 10 min. The cells were incubated in primary antibody solution (1 µg/ml anti-LRF antibody, 1% bovine serum albumin in PBS) for 1h at room temperature and washed three times with PBS. Secondary antibody conjugated to peroxidase was added and incubated for 30 min (InniPress polymerized reporter enzyme staining system, Vector Laboratories). Peroxidase activity was detected by the addition of 3,3-diaminobenzidine for 2 min. Images were captured with a light microscope under a 40x objective (Nikon ECLIPSE TE 2000). Negative controls included the omission of primary antibody and the use of an irrelevant primary antibody (data not shown).

Western blotting
Cells were lysed on ice with RIPA buffer (50mM Tris-Cl, pH 7.5, with 120mM NaCl, 10mM NaF, 10mM sodium pyrophosphate, 2mM EDTA, 1mM Na₃VO₄, 1mM PMSF, 1% NP-40) containing protease inhibitor cocktail (Roche). The amounts of protein in the lysates were determined by Bradford µg of protein was resolved by 8 or 12% sodium dodecyl

Scratch assays
FS090 and SW1353 cells were seeded in 24-well plates at 90% confluency and allowed to adhere overnight. A scratch was made along the center of each well using a 200 µl pipett tip. The wells were then washed twice with PBS to remove loose cells, and fresh medium was added. Photographs were taken at 0 and 24h to assess the ability of cells to migrate and close the gap.

Invasion assays
The invasive potential of wild-type and LRF-depleted chondrosarcoma cells was assessed in 24-well matrigel invasion chambers (BD Biosciences). Briefly, the matrigel inserts and equal numbers of control inserts were prepared as described in the manufacturer’s protocol. FS090 and SW1353 cells (5 × 10⁴ cell/ml in 0.5 ml serum-free media) were added in the upper chambers, and medium (0.75 ml supplemented with 5% fetal bovine serum) was added to the bottom chambers as chemoattractant. After 22h incubation, the non-invasive cells remained at the top chambers were removed by scraping, and the invasive cells at the bottom of the membranes were fixed with 3.7% paraformaldehyde. Cells were then washed twice with PBS and stained with 0.05% crystal violet for 1h at room temperature. The percentage invasion was determined from the ratio of invading cells (matrigel membranes) relative to migrating cells (control membranes) as described in the manufacturer’s protocol.

Annexin V labeling
At 9-day posttransduction, 1 × 10⁴ cells were seeded in 100 mm tissue culture dishes and treated with doxorubicin at one-fifth the IC₅₀ concentration (0.13 µM for FS090 and 0.08 µM for SW1353 cells) for 24h. Doxorubicin-containing medium was replaced with fresh medium and the cells were allowed to grow for an additional 24h. Cells were then double stained for Annexin V and propidium iodide with the Annexin-V-FLUOS staining kit (Roche) following the manufacturer’s protocol. Staining profiles were acquired by flow cytometry (FACS FS-500, Becton Dickinson) and the data analyzed by Flowjo software.

Statistical analysis
Values of all measurements were expressed as the mean ± standard deviation. Statistical comparison was performed by two-tailed Student’s t-test using JMP 9.0 Software (P < 0.05 was considered significant).

Results

Elevated LRF expression is associated with chondrosarcoma malignancy
To assess the presence and extent of LRF in chondrosarcoma, we examined LRF expression in human chondrosarcoma tissue microarrays. A total of three different cases of benign chondroma and 23 cases of chondrosarcoma of varying grades (grade I = 15 cases, grade II = 3 cases and grade III = 5 cases) were examined. Specific nuclear staining of LRF was identifiable under 40x magnification in all three grades of chondrosarcoma, but was absent in sections of chondroma. Positive immunostaining was most intense in grade III specimens (Figure 1A). Additional representative 10x images of benign chondroma and grade I–III chondrosarcoma are shown in Supplementary Figure 1, available at Carcinogenesis Online. Importantly, the percentage of LRF-positive cells and the intensity of the immunostaining were positively correlated with chondrosarcoma malignancy. For example, grade III chondrosarcoma had the highest percentage of LRF-positive cells (range = 44.6–71.2%; mean = 53.2%; Figure 1B), and all five samples of grade III tumor exhibited strong 3+ LRF staining intensity (Figure 1C). In contrast, the percentage of LRF-positive cells was lower in grade I (range = 0–45.9%; mean = 13.3%) and grade II (range= 6.9–36.2%; mean = 30.5%) tumors, and LRF-positive cells were essentially undetectable in benign tumors (Figure 1B and 1C). The LRF expression was significantly different in grade II and grade III chondrosarcoma from benign (P-values < 0.05 and 0.0005, respectively) and also the difference was significant between different grades of chondrosarcomas (P < 0.005). There was a large variation in the percentage of LRF-positive cells (ranging from 0 to 45.9%) and the cellular staining intensity (Figure 1C) among grade I chondrosarcomas, of which four examples are shown in Supplementary Figure 2, available at Carcinogenesis Online. Taken together, our data indicate that elevated LRF expression is associated with increased malignancy of chondrosarcoma.

LRF depletion inhibits proliferation of chondrosarcoma cell lines
Since elevated LRF expression was associated with increased chondrosarcoma malignancy, we next examined the effects of LRF depletion on two grade II human chondrosarcoma cell lines, FS090 (14)
and SW1353 (15). SiRNA against LRF, or against EGFP as control, was stably introduced into these cells by lentiviral transduction. The entire pool of transduced cells was analyzed in all subsequent experiments; the infection efficiency was routinely above 80–90% (data not shown). This procedure eliminated the clonal effect that is often associated with antibiotic selection in establishing stable transfected clones. Six days after transduction, the effective depletion of LRF protein (>80% knockdown) in both cell lines was confirmed by western blot (Figure 2A and 2B insets).

We next determined the effects of LRF depletion on the growth rates of FS090 and SW1353 cells by WST proliferation assays. Depletion of LRF resulted in significant inhibition of cellular proliferation over a period of 4 days in both cell lines (Figure 2A and 2B). The long-term effects of LRF depletion were also examined by colony formation assays over a period of 3 weeks. Following LRF depletion, the ability of these cells to form colonies was significantly impaired (Figure 2C and 2D). Together, these data suggest that LRF is essential for maintaining proliferation of chondrosarcoma cells.

LRF depletion induces G1 cell cycle arrest

We next investigated the mechanism by which LRF regulates cellular proliferation. The cell cycle profiles of wild-type FS090 and SW1353, and cells expressing EGFP- or LRF-siRNA, were determined by flow cytometry. When compared with wild-type and EGFP-siRNA control cells, LRF-siRNA-expressing cells exhibited a significant accumulation of cells in the G1 phase, with a corresponding reduction of cells in the S and G2/M phases (Figure 3). These data suggest that LRF depletion induces G1 arrest and thus explain the drastic reduction in the proliferation rates of these cells observed earlier.

LRF depletion causes senescence and upregulation of p53 and p21

Since LRF depletion induces senescence in mouse embryonic fibroblasts (9), we next performed double staining to simultaneously detect the expression of LRF and the senescence marker β-galactosidase in wild-type and LRF-depleted FS090 and SW1353 cells. As shown in Figure 4A, LRF expression was detected as intense nuclear staining (brown color) in both wild-type and EGFP-siRNA-expressing cells, but not in LRF-siRNA-expressing cells, indicating the effective depletion of LRF by siRNA. Since LRF is mainly located in the nucleus, the faint brown cytoplasmic staining detected in all samples was probably due to non-specific staining, as LRF depletion only specifically removed the intense nuclear stain. Importantly, β-galactosidase activity (blue color) was detected only in LRF-depleted cells, but not in wild-type or control cells. The percentage of β-galactosidase-positive cells were counted in three different fields at 10× magnification (Supplementary Figure 3, available at Carcinogenesis Online). Both wild-type and EGFP-siRNA control cells had <5% senescence cells. In contrast, the percentages of β-galactosidase-positive cells were markedly increased in LRF-depleted FS090 (~50%) and SW1353 (~80%) cells (Figure 4B; Supplementary Figure 3, available at Carcinogenesis Online). These data indicate that LRF depletion causes senescence in chondrosarcoma cells.

LRF is reported to indirectly reduce p53 expression (9), which is important in regulating cell cycle arrest and inducing senescence. To determine whether this pathway is activated in chondrosarcoma,
Fig. 2. LRF depletion suppresses proliferation of chondrosarcoma cell lines. The growth rates of FS090 (A) and SW1353 (B) cells expressing LRF- or EGFP-targeting siRNA were determined by WST assays. The western blots in the insets showed that LRF-siRNA effectively reduced LRF protein expression in both cell lines. The data represented mean ± SD of three independent experiments with each time point measured in triplicates (*P < 0.05). The effects of LRF-siRNA on the abilities of FS090 (C) and SW1315 (D) cells to form colony after 3 weeks were determined by colony formation assays.

Fig. 3. LRF depletion leads to G1 cell cycle arrest. FS090 and SW1353 cells were transduced with lentivirus-expressing EGFP- or LRF-targeting siRNA. Cells were allowed to grow for 8 days and the cell cycle profiles were determined by flow cytometry. The percentage of cells in each cell cycle phase was determined by Flowjo software.
we next examined the protein level of p53 and its downstream
target p21, in LRF-depleted chondrosarcoma cells. The western blot results
demonstrated that p53 was markedly elevated in LRF-depleted FS090 and SW1353 cells (Figure 4C). The expression of p21 was also markedly increased. Collectively, these data suggest that the function of LRF in preventing cellular senescence in chondrosarcoma is, in part, due to its ability to suppress p53 and p21 expression.

LRF depletion decreases cellular migration and invasion

To test whether LRF contributes to tumor malignancy in terms of metastatic potential, we investigated the role of LRF in cell migration using in vitro scratch assays. As shown in Figure 5A, both wild-type and EGFP-siRNA-expressing FS090 and SW1353 cells had similar migratory abilities in monolayer cultures (~70% wound closure after 24 h; Figure 5A, lower panel). In contrast, LRF-depleted cells exhibited a markedly reduction in their abilities to migrate (~17% wound closure for FS090 cells and ~39% for SW1353 cells; Figure 5A, lower panel). These data demonstrate that LRF is important for cellular migration through a 2D environment. We next tested the role of LRF in chondrosarcoma cell migration through a 3D matrigel matrix. The results showed that both wild-type and EGFP-siRNA-expressing FS090 and SW1353 cells had similar invasion capabilities (~90% invasive cells). However, the percentage of invasion was drastically reduced in LRF-depleted cells (~40 and 37% for FS090 and SW1353, respectively, Figure 5B). These results indicate that LRF is important for cellular migration and invasion in vitro and hence may contribute to the metastatic potential of chondrosarcoma.

LRF depletion enhances sensitivity of chondrosarcoma cells to doxorubicin

Previous reports have shown the involvement of p53 and p21 in enhancing the chemosensitivity of cancer cells (16–18). Since LRF is elevated in advanced grade chondrosarcomas and the tumor is highly resistant to chemotherapy, we tested whether LRF depletion and its associated upregulation of p53 and p21 will enhance the chemosensitivity of chondrosarcoma. The proliferation of wild-type and LRF-depleted chondrosarcoma cells was determined by WST assay after treatment with doxorubicin, a common apoptosis-inducing chemotherapeutic agent against sarcomas (19). Cellular sensitivity to doxorubicin was expressed as percentage of cell survival relative to untreated cells. There was a significant increase in doxorubicin sensitivity across all three doses tested in LRF-depleted FS090 cells (Figure 6A, right panel). In the case of SW1353 cells, the increase in doxorubicin sensitivity was significant at 0.02 and 0.39 μM of doxorubicin (Figure 6A, left panel), and trended lower at 0.08 μM. To confirm the enhanced chemosensitivity by LRF depletion, we next determined the percentage of apoptotic cells by Annexin V staining in FS090 and SW1353 cells treated with doxorubicin. As expected, the percentage of cells undergoing apoptosis was drastically increased after LRF depletion in both cell lines (Figure 6B). We conclude that...
Discussion

A major challenge in evaluating chondrosarcoma is the inherent difficulty in distinguishing benign from low-grade tumors, and low-grade from high-grade chondrosarcoma (5). In this study, we demonstrate that chondrosarcoma malignancy coincides with elevated expression of the oncogene LRF (Figure 1). Previous reports have shown elevated LRF expression in lymphomas and in malignant breast and prostate tumors in comparison with their benign counterparts (9–11). These observations, together with its established oncogenic role in other types of tumors, indicate that LRF might be an invaluable diagnosis and prognosis marker to facilitate therapeutic decisions for chondrosarcoma patients. For example, the four cases of grade I chondrosarcoma examined in this study had similar histological appearances but varying degree of LRF expression ranging from 0 to 38.8% (Supplementary Figure 2, available at Carcinogenesis Online). In light of the correlation between elevated LRF expression and increasing grade of chondrosarcoma (Figure 1), we predict that the grade I tumor with high percentage of LRF-positive cells will have the highest chance of progressing into more aggressive tumors; however, further studies will be necessary to determine the correlation between LRF expression and the clinical outcome of these patients.

Previously, we have shown that LRF overexpression inhibits chondrogenic differentiation of mouse mesenchymal stem cells (6). Our present results demonstrated that knockdown of LRF drastically reduces cellular proliferation (Figure 2) by arresting cells at the G1 phase (Figure 3) through increased expression of the major cell cycle regulators p53 and p21 (Figure 4C). The impact of LRF in the regulation of p53 (9) and p21 (20) has been established. Activation of p53-mediated p21 pathway arrests cells at the G1 and G2 checkpoints, and terminates DNA replication (21,22). In addition to cell cycle arrest, LRF depletion also causes senescence in chondrosarcoma cells (Figure 4). Previous reports have shown that upregulation of p53 and p21 is associated with the appearance of the senescence phenotype (23,24). For example, inactivation of the p21 gene prevents senescence in human fibroblasts (25), whereas ectopic overexpression of p21 causes premature senescence (26). In addition, there is ample evidence implicating the involvement of LRF in regulating senescence in other cell types (8,27–29).

Besides LRFs role in enhancing cellular proliferation and preventing senescence through p53 inhibition, we have demonstrated for the first time the importance of LRF in cellular migration and invasion (Figure 5). This result may explain the high levels of LRF expression in advanced chondrosarcoma, which has higher metastatic potential. Cell migration and invasion are crucial steps during cancer progression that can be affected by a variety of adhesion molecules such as cadherins, integrins, matrix metalloproteases, and the chemokines that controls their expression (30). One of the chemokines, the stromal-derived factor-1 (SDF-1) is involved in metastatic cancers (31). Interestingly, p53 functions to inhibit SDF-1 expression, leading to reduced tumor cell migration and invasion (32). This finding is in agreement with our data showing that LRF depletion, which leads to p53 upregulation, results in decreased cellular invasion of chondrosarcoma cells. Although, whether this is SDF-1-dependent remains to be tested in chondrosarcoma. Besides SDF-1, there are plenty of evidence suggesting the involvement of p53 and its regulated genes, such as p21, in cell adhesion, migration and cytoskeletal organization (reviewed in ref. 33). These reports indirectly suggest that the reduction of migration and invasion in LRF-depleted cells is probably to be connected to p53 and p21.

Drug resistance is a major problem in the treatment of chondrosarcomas, as currently there is no effective therapeutic agent for this disease. Only a limited number of studies have explored new
therapeutic approaches and examined the mechanisms that contribute to the chemoresistance of chondrosarcomas. These include the reports that the chemoresistance of chondrosarcoma is due to the increased expression of the multi-drug resistance factor p-glycoprotein (34–36), and that siRNA-mediated silencing of antiapoptotic genes enhances chemoresistance of chondrosarcoma cells (36–38). Our results demonstrate the importance of LRF as a survival factor that prevents premature senescence in human chondrosarcoma. Our observation that LRF depletion sensitizes chondrosarcoma cells to doxorubicin and enhances their apoptosis (Figure 6) can be explained by the increase in p53 expression and senescence in these cells. A role of increased p53 expression in sensitization of non-small cell lung cancer to chemotherapeutic agents has been reported previously (17). Furthermore, induction of senescence, through inhibition of cathepsin L and stabilization of p21, reduces drug resistance in neuroblastoma, osteosarcoma and leukemia cell lines (29). In light of our findings, LRF appears to be a key determinant in chondrosarcoma survival and could be a potential target for new therapeutic approach.

In summary, our data demonstrate that elevated LRF expression is associated with advanced grade chondrosarcoma, and that LRF is an anti-senescent factor important for the survival, invasion and chemoresistance of chondrosarcoma cells. Our study thus demonstrates the potential values of LRF as both a diagnostic marker and therapeutic target for chondrosarcoma.

Supplementary material

Supplementary Figures 1–3 can be found at http://carcin.oxfordjournals.org/

Funding

Chapman Endowed Fund to P.D.; departmental startup funds to J.Y. and D.R.H.
Acknowledgements

We thank Dr Robert Tamurian for helpful suggestion and Dr Dariusz Borys for assistance in the evaluation of chondrosarcoma tissue microarrays.

Conflict of Interest Statement: None declared.

References


Received February 28, 2012; revised July 11, 2012; accepted July 22, 2012