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Targeting connective tissue growth factor (CTGF) in acute lymphoblastic leukemia pre-clinical models: anti-CTGF monoclonal antibody attenuates leukemia growth

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Abstract

Connective tissue growth factor (CTGF/CCN2) is involved in extracellular matrix production, tumor cell proliferation, adhesion, migration and metastasis. Recent studies have shown that CTGF expression is elevated in precursor-B acute lymphoblastic leukemia (ALL) and that increased expression of CTGF is associated with inferior outcome in B-ALL. In this study, we characterized the functional role and downstream signaling pathways of CTGF in ALL cells. First, we utilized lentiviral shRNA to knock-down CTGF in RS4;11 and REH ALL cells expressing high levels of CTGF mRNA. Silencing of CTGF resulted in significant suppression of leukemia cell growth compared to control vector, which was associated with AKT/mTOR inactivation and increased levels of cyclin-dependent kinase inhibitor p27. CTGF knockdown sensitized ALL cells to vincristine and methotrexate. Treatment with an anti-CTGF monoclonal antibody, FG-3019, significantly prolonged survival of mice injected with primary xenograft B-ALL cells when co-treated with conventional chemotherapy (vincristine, L-asparaginase and dexamethasone). Data suggest that CTGF represents a targetable molecular aberration in B-ALL, and blocking CTGF signaling in conjunction with administration of chemotherapy may represent a novel therapeutic approach for ALL patients.

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STATEMENT OF AUTHORSHIP

H.L., V.L.B., B.K., Y.S., Y.C., designed and performed the research and analyzed the data; K.K. analyzed the data and wrote the manuscript; S.S. provided anti-CTGF monoclonal antibody, contributed to discussion and edited the paper; D.A.T. and H.K. provided primary patients' samples, analyzed the data and contributed to discussion; R.B.L. established human primary pre-B-ALL ALL xenografts and analyzed the data; M.A. designed the research and analyzed the data; M.K. initiated, supervised the research, analyzed the data and wrote and edited the paper.

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Keywords

ALL; CTGF; apoptosis

Introduction

Connective tissue growth factor (CTGF or CCN2) is an extracellular matrix-associated molecule and a member of the CCN family, which includes cysteine-rich protein 61 (Cyr61 or CCN1), CTGF/CCN2, nephroblastoma overexpressed protein (Nov or CCN3), Wnt-inducible secreted protein-1 (WISP-1 or CCN4), WISP-2 (CCN5) and WISP-3 (CCN6). The CCN family members possess an NH2-terminal signal peptide indicative of secreted proteins. CTGF is reported to interact with various proteins including integrins, bone morphogenetic proteins, transforming growth factor (TGF)-β, aggrecan, matrix metalloproteinases, fibronectin, perlecan, vascular endothelial growth factor (VEGF) and low-density lipoprotein receptor-related proteins [1-3]. CTGF is involved in extracellular matrix production, cell proliferation, cell survival, adhesion, migration, and metastasis [1-3]. None of the *in vivo* activities of CTGF have been unequivocally attributed to specific interactions, suggesting that CTGF might mediate its effects through multiple mechanisms.

CTGF over-expression has been associated with tumor progression and/or poor prognosis of solid cancers including breast cancer, glioblastoma and esophageal cancer [4-8]. In pancreatic cancer, CTGF is a critical regulator of tumor growth, and CTGF-specific antibody attenuates tumor growth and metastases *in vivo* [7, 8]. On the other hand, increased CTGF expression has been correlated with improved prognosis in chondrosarcoma patients and in patients with lung cancer [9, 10]. CTGF has been reported to confer anti-apoptotic properties and chemoresistance in cancer [11-14]. In hematological malignancies, elevated CTGF expression has been frequently and exclusively detected in precursor-B acute lymphoblastic leukemia (ALL) [15-18]. CTGF is poorly expressed in normal peripheral blood and hematopoietic bone marrow cells, AML or T-lineage ALL, while 70–80% of precursor-B ALL samples over-express CTGF [15-18]. High expression of CTGF has been associated with poor outcome in precursor-B ALL patients [18, 19]. It is thought that CTGF functions in a cell-type-specific, context-dependent manner and is a potential therapeutic target for some malignancies including precursor-B ALL. However, up until now, a direct role for CTGF in tumor suppression or progression has not been investigated in leukemias or with therapeutic agents with the capacity to inhibit CTGF function *in vivo*.

In this study, we characterized expression and function of CTGF in ALL cells and investigated the anti-leukemia efficacy of the human anti-CTGF monoclonal antibody FG-3019 (FibroGen, San Francisco, CA).

Methods

Reagents

CTGF monoclonal antibody—FG-3019 is a human $IgG_{1\kappa}$ monoclonal antibody recognizing domain 2 of human and rodent CTGF, provided by FibroGen (San Francisco, CA). In the indicated experiments, whole molecular human IgG, purified from serum (Jackson ImmunoResearch), was used as the control.

Cell lines, primary samples and cultures—RS4;11, REH, Raji and Jurkat cell lines were purchased from the American Type Culture Collection. NALM-6 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen. Cell lines were maintained in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS). RS4;11,

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REH and NALM-6 are derived from precursor-B ALL patients, the mature B-cell Raji from a Burkitt lymphoma patient and Jurkat from a T-ALL patient. Human primary pre-B-ALL ALL xenografts (ALL-2 and ALL-10) were generated from pediatric ALL xenografts propagated in mice [20]. The clinical information has been also described [20]. Normal bone marrows were obtained after informed consent in accordance with institutional guidelines set forth by M. D. Anderson Cancer Center and the Declaration of Helsinki. Mononuclear cells were purified by Ficoll-Hypaque (Sigma Chemical Co., St. Louis, MO) density-gradient centrifugation, and non-adherent cells were resuspended in RPMI 1640 medium supplemented with 10% FBS at a density of 5×10^5 cell/mL. Cells were counted with a Vi-Cell Counter (Beckman Coulter, Brea, CA).

Western blot analysis—Equal amounts of protein lysate were separated by SDS-PAGE. Proteins were transferred to nitrocellulose membrane, immunoblotted with primary antibodies followed by secondary antibodies (LI-COR Biosciences, Lincoln, NE), and detected by the Odyssey imaging system (LI-COR Biosciences). The following antibodies were used: goat polyclonal anti-CTGF (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit polyclonal anti-AKT (Cell Signaling Technologies Beverly, MA); rabbit monoclonal anti-phospho-AKT (Ser^{473}) (Cell Signaling Technologies); rabbit monoclonal anti-phospho-S6 Ribosomal Protein (S6RP) (Cell Signaling Technologies); rabbit polyclonal anti-phospho-S6RP ($Ser^{240/244}$); rabbit polyclonal anti-4EBP1 (Cell Signaling Technologies); rabbit monoclonal anti-phospho-4EBP1 ($Thr^{37/46}$) (Cell Signaling Technologies); mouse monoclonal anti-p27 (BD Biosciences, San Jose, CA); goat polyclonal anti-cIAP1 (R & D Systems, Minneapolis, MN), rabbit polyclonal anti-BCL-X_L (BD Biosciences), rabbit polyclonal anti-BIM (Millipore, Billerica, MA), and mouse monoclonal anti-GAPDH (Millipore).

CTGF knockdown by lentiviral transduction—RS4;11 and REH cells were transduced with lentiviruses encoding either CTGF-specific shRNA (RHS3979-9629133; Open Biosystems, Lafayette, CO) or empty vector (RHS4080). Lentiviral infections were carried out according to the standard procedures for silencing experiments. In brief, 293T cells were co-transfected with viral packaging vectors pMD2.G and psPAX2 (Addgene, Cambridge MA), along with a lentiviral construct expressing either a specific *CTGF*-shRNA or the empty vector as control, using JetPrime transfection reagent (Polyplus-transfection, New York, NY) according to the manufacturer's protocol. The transfection medium was replaced after 12 hours with fresh DMEM/10% FBS, and 48 hours later the viral supernatants were collected and used for infections. ALL cells were then transduced with a viral supernatant derived from empty lentiviral vector or the *CTGF*-shRNA–expressing vector. After incubation for 48 hours, the viral supernatant was replaced with complete cell culture medium containing 2 μg/ml puromycin (Invitrogen, Carlsbad, CA) for selection. ALL cells were cultured for 5 days in selection medium, which was then replaced with complete cell culture medium and used in the experiments.

Quantitative real-time PCR—cDNA were obtained by reverse transcription of 1 μg of DNase-treated total RNA from each sample using random hexamer priming in 20 μl reactions. The mRNA expression levels of CTGF and ABL1 were quantified using *Taq*Man gene expression assays (CTGF: Hs01026927_g1, ABL1: Hs01104728_m1, Applied Biosystems, Foster City, CA; H) on a 7900HT Fast Real-Time PCR System.

Cell cycle analysis—Cells were fixed in 70% ice-cold ethanol and stained with 25 μg/ml propidium iodide solution (Sigma Chemical). The DNA content was determined using a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose,

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CA). Cell cycle distribution was analyzed using ModFit LT software (Verity Software House, Topsham, ME).

Apoptosis assay—Leukemia cells were treated with different chemotherapy drugs for 48 hours. The apoptotic leukemia cells were detected by Annexin V flow cytometry. Briefly, cells were washed twice with binding buffer (10 mM HEPES, 140 mM NaCl, and 5 mM $CaCl_2$ at pH 7.4; Sigma Chemical) and incubated with a 1:50 solution of FITC-conjugated Annexin V (Roche Diagnostic, Indianapolis, IN) for 15 minutes at room temperature. Stained cells were analyzed by flow cytometry and membrane integrity was simultaneously assessed by PI exclusion.

Animal experiments—All animal work was carried out in accordance with a protocol approved by the institutional animal care and use committee of MD Anderson Cancer Center. NOD/SCID mice (ALL-2) or NSG mice (ALL-10) were inoculated with 3.5×10^6 human primary pre-B-ALL ALL xenografts (ALL-2 and ALL-10) [20]. Treatment commenced when the percentage of human $CD45^+$ cells in peripheral blood exceeded the median value of >1% for the complete cohort. In the experiment using ALL-2 cells, FG-3019 and control human IgG (10 mg/kg, twice weekly, i.p) were combined with conventional chemotherapy (VXL, vincristine, once a week, i.p., 0.15 mg/kg; L-asparaginase, 5 times a week, i.p., 1000 U/kg; dexamethasone, 5 times a week, i.p., 5 mg/kg) for 5 weeks total beginning at day 84 after leukemia cell injection (10 mice/group). FG-3019 and human IgG doses were increased to 20 mg/kg (twice weekly, i.p.) from week 6 – week 11 in the groups that also received VXL from week 1-5. For ALL-10 cells, FG-3019 and control human IgG (30 mg/kg, twice weekly, i.p) were combined with VXL as above for 3 weeks total beginning at day 3 following leukemia cell injection (8 mice/group). Mice were sacrificed if they became morbid or had a weight loss of 20% or greater.

Statistical analysis—Statistical analysis was performed using the 2-tailed Student's t test or Mann-Whitney test if appropriate. Survival curves were created by the Kaplan-Meier method and compared by the log-lank test. Results were considered statistically significant at p-values < .05. Unless otherwise indicated, average values were expressed as mean ± SD.

Results

CTGF is highly expressed in precursor-B ALL cells

We first investigated CTGF mRNA expression in ALL cell lines. RS4;11 and REH expressed CTGF mRNA (479.3 ± 37.2 and 57.3 ± 5.9 per 100 copies of ABL1, respectively) (Fig. 1a). CTGF expression was not detected in NALM-6, Raji or Jurkat cells. In 11 primary precursor-B ALL patient samples (8 xenograft B-ALL cells [20] and 3 primary samples), CTGF mRNA levels varied from 68.7 to 12731 per 100 copies of ABL1 (Fig. 1b). The median mRNA expression in patient blasts (3823) was 8 times higher than CTGF mRNA levels in RS4;11 cells, suggesting an active involvement of CTGF in primary precursor-B ALL blasts.

CTGF knockdown exhibits anti-leukemia effects in precursor-B ALL

To investigate the biological consequences of CTGF expression in ALL cells, RS4;11 and REH cells were infected with lentivirus encoding either empty vector (RS4;11-EV) or CTGF-specific shRNA (RS4;11-shCTGF). CTGF-specific shRNA led to reduced basal CTGF mRNA expression by 65% in RS4;11 cells (Fig. 2a) and by 55% in REH cells (data not shown). We observed retarded growth of CTGF knockdown cells as compared with control cells (Fig. 2b, Supplementary Fig. S1). CTGF knockdown caused significant inhibition of the G_1/S transition, with accumulation of cells in the G_1 phase (Supplemental

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Fig. S1). It has been reported that CTGF stimulates AKT-mediated reduction of p27, which is a key regulator of G_1/S transition [21, 22]. Consistent with previous reports, CTGF knockdown resulted in decreased levels of phospho-AKT, downstream targets of mTOR phospho-S6RP and phospho-4EBP1 and increased levels of p27 (Fig. 2c), which could cause G1 cell cycle arrest. Levels of anti-apoptotic proteins cIAP1 and BCL-X_L did not change. CTGF knockdown led to increased levels of the pro-apoptotic BCL-2 family protein BIM.

To investigate if modulation of CTGF expression can sensitize ALL cells to conventional chemotherapeutic agents, RS4;11-EV/-shCTGF and REH-EV/-shCTGF cells were treated for 48 hours with vincristine (VCR) and methotrexate (MTX) *in vitro*. CTGF knockdown was associated with modest phosphatidylserine externalization in both cells (Fig. 3). On the contrary, shCTGF-expressing cells were more susceptible to VCR and MTX, implying that reduced CTGF expression may sensitize ALL cells to these agents.

Anti-CTGF monoclonal antibody attenuates tumor growth of precursor-B ALL in vivo

We next investigated the anti-leukemic efficacy of the monoclonal anti-CTGF antibody FG-3019 in ALL models. FG-3019 failed to affect cell growth in liquid cultures or chemosensitivity of ALL cells cultured *in vitro*, consistent with previously reported findings in pancreatic cancer cell models [23]. Since CTGF most likely plays a role in the interaction of ALL cells with cells of the bone marrow microenvironment, we examined efficacy of FG-3019 in the *in vivo* ALL model. The anti-leukemic effects of FG-3019 in combination with an induction-type regimen consisting of vincristine, L-asparaginase and dexamethasone (VXL) [24] were evaluated in mice injected with cells from two pediatric ALL patients propagated *in vivo* (ALL-2 and ALL-10) [20]. ALL-2 and ALL-10 expressed high levels of CTGF mRNA at 7,248 and 12,731 per 100 copies of ABL1, respectively (Fig. 1B). While ALL-10 was far more aggressive than ALL-2, FG-3019 alone did not affect leukemia progression in either of these primary xenograft models (ALL-2: $p = 0.61$; ALL-10: $p = 0.84$) (Fig. 4). VXL treatment significantly extended mouse survival compared to IgG control in ALL-2 ($p = 0.023$) but not in ALL-10 ($p = 0.32$). The combination of FG-3019 with VXL significantly prolonged mouse survival compared with IgG with VXL in both cases (ALL-2, $p = 0.027$; ALL-10, $p = 0.033$).

Discussion

Recent studies have reported increased expression of CTGF in precursor-B ALL, which was significantly associated with inferior outcome [18, 19]. In this study, we characterized mRNA expression and function of CTGF in ALL cell lines and in samples from primary ALL patients. In accordance with previous reports, precursor-B ALL cell lines and patient samples expressed CTGF mRNA. CTGF mRNA expression was detected in 2 of 3 precursor-B ALL cell lines (positive for RS4;11 and REH and negative for NALM-6) and in all 11 primary precursor-B ALL patient samples, raising the possibility that CTGF is actively involved in this specific subtype of ALL. Notably, the majority of primary ALL cells express higher CTGF levels than cell lines, and the expression in the xenograft cells passaged *in vivo* (ALL-2 and ALL-10) appear to be more closely aligned with the primary samples than with cell lines. This suggests that CTGF expression may be important in the context of the bone marrow microenvironment where the cells are propagated.

The mechanisms of CTGF over-expression in B precursor ALL remain unknown. The CTGF gene is located on Chromosome 6q23, and trisomy 6 or 6q23 abnormalities are rare in ALL (Atlas of Genetics and Cytogenetics in Oncology and Haematology. URL http://AtlasGeneticsOncology.org). CTGF gene mutations or amplification have not been reported. Alternatively, CTGF mRNA could be upregulated downstream of specific microRNA

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overexpression [25, 26]. As such, CTGF is a known predicted target of miR-18a, miR-133, miR-30, miR-17-92 microRNA cluster, from which miR-17-92 cluster and miR-30e-5p were found to be upregulated in ALL. Further, CTGF is a well-known immediate early gene that is potently induced by a variety of stimuli that normally regulate extracellular matrix deposition, tissue remodeling, and neovascularization, including platelet-derived growth factor, TGF-β, basic fibroblast growth factor and VEGF; however, the role of these factors in the biology of human ALL has not been defined. Interestingly, CTGF expression can be induced by hypoxia [27-29]. Hypoxia occurs along with bone marrow infiltration by precursor-B ALL cells [30]. Hypoxia has been reported to induce CTGF via the HIF-1 pathway [27, 28]. Hypoxia has also been reported to increase CTGF expression by 3′-untranslated region-mediated increases in CTGF mRNA stability [29]. Thus, we propose the hypothesis that precursor-B ALL cells generate a hypoxic microenvironment and thereby induce CTGF to support their survival advantage and proliferation.

We found that CTGF knockdown in precursor-B ALL cells leads to reduced cell proliferation with G1 arrest. Although identification of downstream mediators of CTGF that promote cell survival and proliferation requires further investigation, our data point to the relevance of AKT/mTOR activation, affecting protein expression of cell cycle regulator p27 and pro-apoptotic BH3 protein BIM. Possibly as a result of blockade of these pro-survival pathways, CTGF knockdown sensitized precursor-B ALL cells to chemotherapeutic agents VCR and MTX. These findings suggest that CTGF is critically involved in ALL progression and is a potential therapeutic target for ALL.

In addition to autocrine effects of CTGF on ALL cell signaling, CTGF is known to directly bind to several integrin extracellular matrix receptors and regulate cell adhesion and migration of several cell types [31]. Integrin $\alpha_{IIb}\beta_{3a}$ (CD41/CD61), a CTGF binding partner, has recently been identified on a subpopulation of primitive HSC, both human and murine [32, 33], and on most hematopoietic progenitor cells derived *in vitro* from murine embryonic stem cells [34]. In our cell line studies we failed to detect significant effects of FG-3019 on CXCL12-induced migration or adhesion of ALL cells to fibronectin *in vitro*, however this may reflect our inability to recreate an appropriate microenvironment that mimics the conditions of the *in vivo* setting. Notably, CTGF was recently identified as the top upregulated gene in a large cohort of primary B-ALL samples by microarray analysis, and an *in silico* functional analysis suggested the linkage to pathways regulating leukemia-microenvironment interactions [16].

To determine potential therapeutic efficacy of CTGF blockade in the *in vivo* leukemia models, we investigated the effects of the human anti-CTGF monoclonal antibody FG-3019 in the mouse model of precursor-B ALL, using patient-derived leukemia cells. A mouse survival study showed beneficial results of conventional chemotherapy when combined with FG-3019 in primary human ALL xenografts. FG-3019 has been evaluated in several phase 1 clinical studies in patients with diabetic kidney diseases [35], idiopathic lung fibrosis and pancreatic cancer and is currently in phase 2 testing in idiopathic lung fibrosis (NCT01262001) and chronic hepatitis B infection-related liver fibrosis (NCT01217632). The phase 1 study of FG-3019 in combination with Gemcitabine and Erlotinib in patients with advanced pancreatic cancer (NCT01181245) has completed patient enrollment, and results of interim analysis demonstrated impressive PET responses at the highest dose in some patients [36]. It is noteworthy, that at least in pancreatic cancer, CTGF is expressed not only in the cancer cells, but also in the tumor stroma and is most abundant in pancreatic stellate cells within the stroma [37]. CTGF acts as both an autocrine and paracrine factor but as an ECM-associated factor, may exert it’s effects locally. It was therefore proposed that the efficacy of FG-3019 in this tumor type could be partly attributable to the interference with tumor-associated stroma formation and function, including pancreatic stellate cell

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activation, thereby attenuating collagen I deposition, suppressing further stromal interactions with the tumor, and decreasing stroma-derived tumorigenic factors, such as VEGF-A. Although the effects of CTGF blockade on bone marrow microenvironment remains to be further investigated, CTGF was shown to be upregulated in leukemia inhibitory factor-stimulated stroma cultures, which in turn augmented their ability to support hematopoiesis [38]. Our data indeed demonstrate high expression of CTGF in bone marrow-derived mesenchymal stem cells [39], indicating a potential role of CTGF in leukemia-stroma interactions. Hence, CTGF may represent a unique target functionally important for both, leukemia cell survival and microenvironment-mediated chemoresistance. In summary, our data demonstrate that autocrine CTGF is highly expressed in pre B-ALL cell lines and primary ALL samples and represents a potential druggable target for therapies blocking CTGF signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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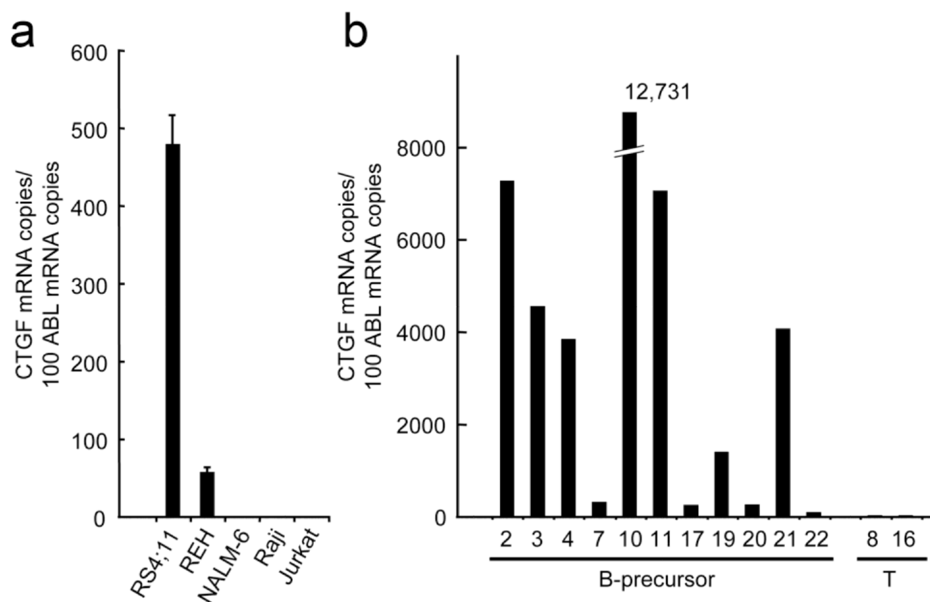

Figure 1. CTGF is highly expressed in precursor-B ALL cells

(A, B) Real-time PCR measurement of steady state expression of *CTGF* in ALL cell lines (A) and patient precursor-B ALL (B precursor) and T-ALL (T) samples (B). The abundance of mRNA was normalized to that of ABL1. Results are expressed as mean ± SD of triplicate measurements in cell lines and mean of duplicate measurements in patient samples. Precursor-B ALL cells expressed high levels of CTGF mRNA.

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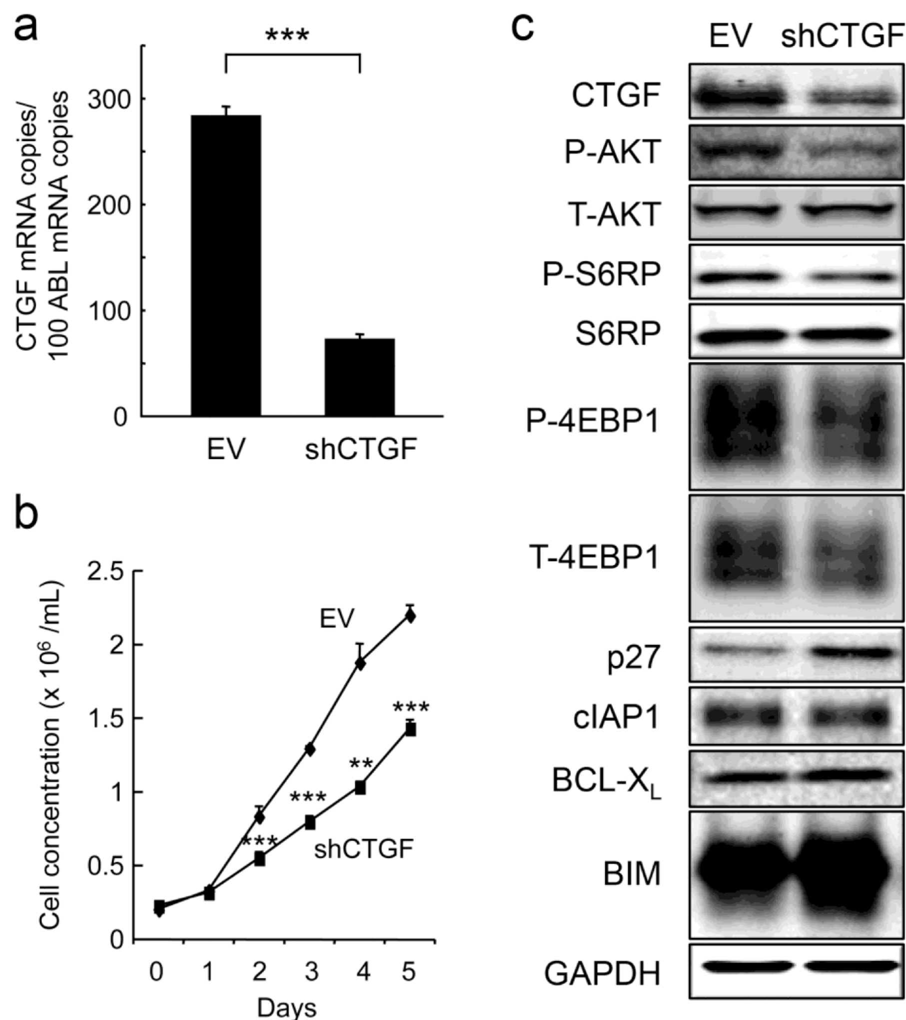

Figure 2. CTGF knockdown inhibits ALL cell proliferation *in vitro*

(A, B) CTGF expression levels (A) and growth curves (B) of RS4;11 cells expressing either empty vector (EV) or CTGF shRNA (shCTGF). The abundance of mRNA was normalized to that of ABL1. Cell proliferation was analyzed by counting absolute cell numbers with a Vi-Cell XR cell counter. Results are expressed as mean ± SD of triplicate measurements. Statistical significances are denoted as follows: $^{**}P < 0.01$, $^{***}P < 0.001$. (C) CTGF knockdown led to inactivated AKT/mTOR components and increased levels of p27. Protein expression was determined by Western blotting.

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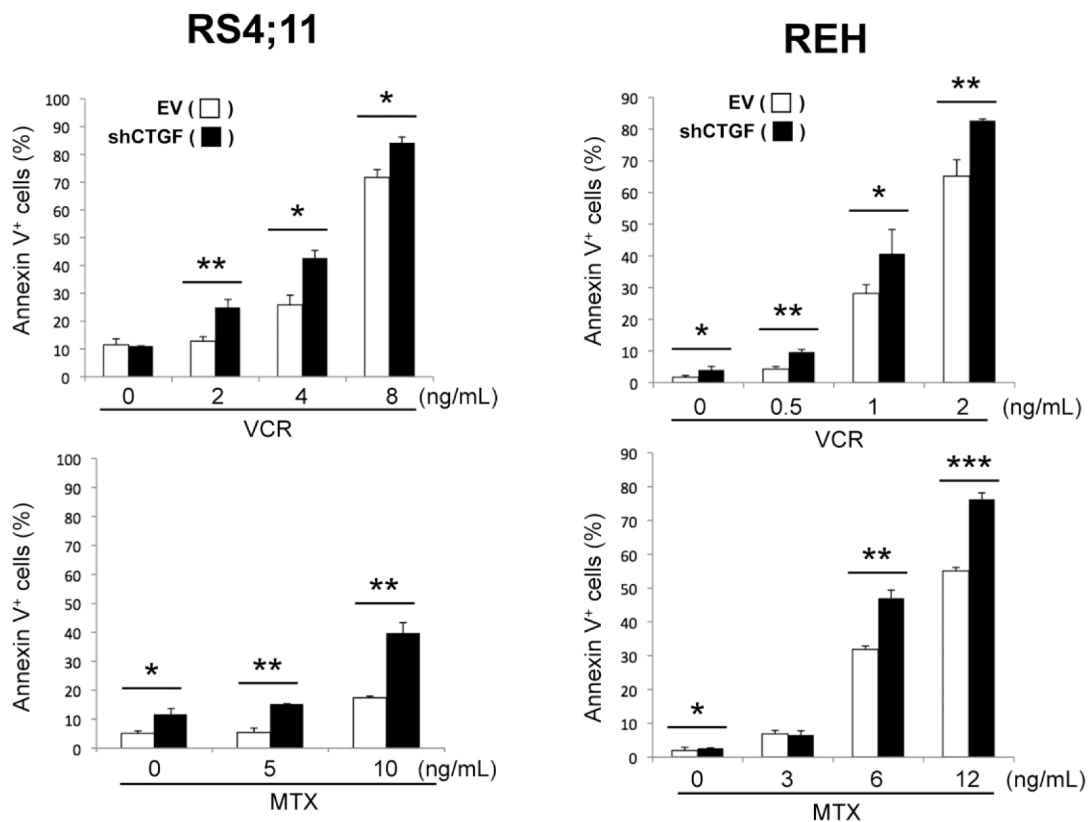

Figure 3. CTGF knockdown enhances chemotherapy-induced apoptosis

RS4;11 and REH cells expressing either empty vector (EV) or CTGF shRNA (shCTGF) cells were treated for 48 hours with the indicated concentrations of vincristine (VCR) and methotrexate (MTX), and Annexin V-positive fractions were measured. Results are expressed as mean ± SD of triplicate measurements. Statistically significant differences are denoted as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

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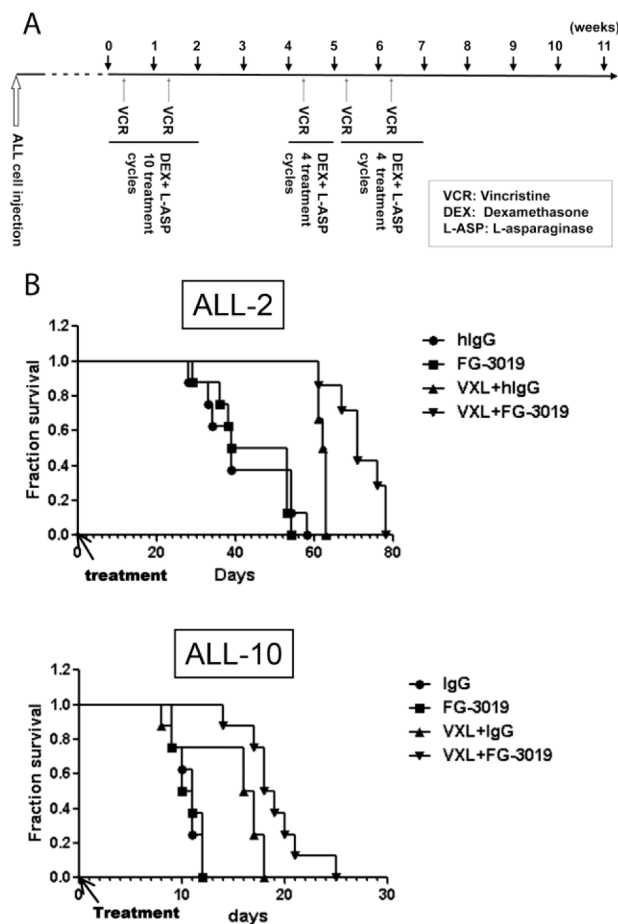

Figure 4. FG-3019 significantly prolongs overall survival in patient-derived human ALL xenograft models when combined with conventional chemotherapy

(A) Schematic representation of the *in vivo* xenograft experiment and chemotherapy/ FG-3019 administration. (B) Overall survival of control (hIgG-treated) and FG-3019-treated mice with or without conventional chemotherapy (vincristine, L-asparaginase and dexamethasone, VXL) in the ALL-2 and ALL-10 xenograft model. X axis, days post initiation of treatment (day 84 for ALL-2, day 3 for ALL-10).