WT1 regulates cyclin A1 expression in K562 cells

SONY PANDEY[1], MUSTAFA MOAZAM[2], NIRMALA GHIMIREY[1], STEVEN J. KUERBITZ[2] and GAIL C. FRAIZER[1,3]

[1]Department of Biological Sciences, Kent State University, Kent, OH 44242; [2]Akron Children's Hospital, Akron, OH 44308; [3]School of Biomedical Sciences, Kent State University, Kent, OH 44242, USA

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Abstract. The restricted expression of Wilms tumor 1 (WT1) and cyclin A1 (CCNA1) in normal tissues, as opposed to their abnormal expression in leukemia demonstrates the applicability of WT1 and CCNA1 as cancer antigens for immunotherapy, and as markers for prognosis and relapse. In this study, the WT1 and CCNA1 mRNA levels were found to be elevated in bone marrow samples from pediatric acute promyelocytic leukemia (APL or AML-M3) patients, and to be quite varied in pediatric acute lymphocytic leukemia (ALL) patients, compared to non-leukemic bone marrow controls. Consistent with the observed upregulation of both WT1 and CCNA1 in APL, WT1 overexpression elevated the CCNA1 mRNA levels in K562 leukemia cells. Treatment with curcumin decreased the WT1 levels in K562 cells, and also decreased CCNA1 protein expression. The examination of the CCNA1 promoter identified potential canonical WT1 binding sites within the 3-kb region upstream of the transcription start site. Chromatin immunoprecipitation and luciferase reporter assays confirmed WT1 binding and the activation of the CCNA1 promoter. Furthermore, the GC-rich core CCNA1 promoter region provided additional non-canonical WT1 activation sites, as revealed by promoter assays. The importance of the GC-rich core region of the CCNA1 promoter was confirmed by treating the K562 cells with mithramycin A, which blocks the binding of zinc finger transcription factors to GC-rich sequences. Mithramycin A subsequently suppressed both CCNA1 promoter activity and protein expression in the K562 cells. Taken together, the data from the WT1 overexpression, and curcumin and mithramycin A treatment experiments, as well as those from chromatin binding assays, along with inferences from patient RNA analyses, establish a plausible link between WT1 and CCNA1, and support the functional significance of an elevated WT1 expression in leukemia, which may also affect CCNA1 expression.

Correspondence to: Dr Gail C. Fraizer, Department of Biological Sciences, Kent State University, 1275 University Esplanade, Kent, OH 44242, USA
E-mail: gfraizer@kent.edu

Abbreviations: ALL, acute lymphocytic leukemia; AML, acute myelocytic leukemia; MRD, minimal residual disease; IRB, Institutional Review Board; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; VEGF, vascular endothelial growth factor; ZF, zinc finger; BM, bone marrow; HSPCs, hematopoietic stem and progenitor cells; NBM, non-leukemic bone marrow; GFP, green fluorescent protein; RLU, relative light units; ChIP, chromatin immunoprecipitation; PVDF, polyvinylidene fluoride; DMSO, dimethyl sulfoxide; IF, immunofluorescence; DAPI, 4',6-diamidino-2-phenylindole; MTT, 3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; SP1, specificity protein 1; EGR1, early growth response protein 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase

Key words: Wilms tumor 1, cyclin A1, acute lymphocytic leukemia, acute myelocytic leukemia, pediatric leukemias

Introduction

Although a wide array of genes has been studied as potential prognostic markers in leukemia, a clear mechanistic understanding has not yet been established. A previous study seeking markers of minimal residual disease (MRD) in acute myeloid leukemia (AML) demonstrated that the combined expression of 3 genes [Wilms tumor 1 (WT1), cyclin A1 (CCNA1) and hemoglobin gamma 2 (HBG2)] could predict MRD in pre- and post-stem cell transplantation setting (1). In normal tissues, the expression levels of WT1 and CCNA1 are limited (2,3); however, they are found elevated in bone marrow (BM) samples of adult and pediatric AML cases (4) and their increased expression is associated with poor clinical outcomes (5,6). Both genes are, therefore, excellent candidates for leukemia/MRD therapy and are leading antigen candidates investigated for AML immunotherapy (7,8).

WT1 is a well-established prognostic and MRD marker in leukemia, as elevated WT1 levels return to normal when patients achieve continuous complete remission (9). However, WT1 may not be overexpressed in all leukemias, and the inclusion of additional gene markers, such as CCNA1 in PCR-based assays could improve predicted relapse risk stratification in patients (10), as an increased CCNA1 expression has been shown to be associated with a poor prognosis of AML (6) and pediatric acute lymphocytic leukemia (ALL) patients (11).

WT1 encodes a zinc finger (ZF) transcription factor that regulates genes, such as vascular endothelial growth factor

(VEGF) and E-cadherin, that play important roles in normal development, as well as in cancer (12-14). Common deletions of the WT1 gene identified in patients with Wilms' tumor involve the loss of all or part of the ZF domain that is required for DNA and RNA binding (15). Although WT1 has been shown to transcriptionally regulate the expression of cyclin D1 in non-small cell lung cancer cells (16) and that of cyclin E in murine 32D cells (17), the regulation of CCNA1 by WT1 has not yet been investigated, at least to the best of our knowledge. By contrast, there has been a report that WT1 isoform ratios were altered in U937 cells overexpressing CCNA1 (particularly enhancing the exon 5 retaining isoforms) (18).

CCNA1 is an unusual cyclin in that it is typically expressed in spermatocytes and hematopoietic progenitor cells (3). In spermatocytes, CCNA1 regulates the M phase of the meiotic cell cycle and CCNA1 null male mice are infertile (19). In the BM, CCNA1 has been shown to regulate pathways that enhance the interaction of hematopoietic stem and progenitor cells (HSPCs) with the BM microenvironment. Indeed, both CCNA1 and VEGFR1 are required for the normal homing of HSPCs in their BM niches (20).

In addition to a potential role in homing, CCNA1 has also been described as a regulator of cell cycle control (21,22). The upregulation of CCNA1 has been shown to promote the proliferation of K562 cells *in vitro* (23) and to promote breast tumorigenesis *in vivo* (24). WT1 has also been shown to be involved in the cell cycle and proliferation. The upregulation of WT1 expression has been shown to promote the proliferation of non-small cell lung cancer cell lines (16) and, conversely, the suppression of WT1 expression has been shown to inhibit the growth of leukemia cell lines (25,26). These findings link WT1 and CCNA1 expression to the proliferation of leukemia cells. Additionally, CCNA1 expression has been linked to VEGF expression in hormone-dependent prostate and breast cancer cells (27-29). Tissue microarray analysis has revealed the elevated expression of CCNA1 and VEGF in prostate cancer tissues, compared to adjacent benign tissues or benign prostatic hyperplasia (27,30). Similarly, WT1 expression has been shown to be elevated in prostate cancer epithelium compared to adjacent 'normal' stromal tissues (31).

In this study, we wished to determine whether the expression of WT1, CCNA1 and VEGF is elevated in pediatric ALL and pediatric acute promyelocytic leukemia (APL or AML-M3) BM samples. Additionally, we wished to determine whether WT1 transcriptionally regulates CCNA1 and VEGF expression in K562 leukemia cells. Our findings suggest a possible role for WT1 as a DNA binding transcription factor which upregulates CCNA1 expression in leukemia and thereby potentially affects HSPC interactions within their BM niche.

Materials and methods

Patient samples. Diagnostic BM aspirates from 20 pediatric ALL patients were obtained in accordance with Akron Children's Hospital Institutional Review Board (IRB) (Akron, OH, USA) guidelines. Ten morphologically normal non-leukemic BM (NBM) samples obtained from patients with non-leukemic disorders (e.g., solid tumor staging evaluation) were used as controls. NBM controls were bone marrow-derived mononuclear cells (isolated from non-neoplastic bone marrow by Ficoll Hypaque gradient centrifugation). The samples that exhibited high Cq values for GAPDH or 18s RNA, indicative of poor RNA quality, were omitted from the analysis; thus, 1 ALL and 2 control samples were excluded. The subtypes are listed in Table I, although the majority (16 samples) were classified as early-B ALL. RNA samples from 10 pediatric APL (or AML-M3) patients were obtained from the Children's Oncology Group (COG). COG is the pediatric cancer network component of the NCI National Clinical Trials Network (www.childrensoncologygroup.org). No data of the patient characteristics were available for this cohort.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from the BM samples using the 5 PRIME PerfectPure RNA Blood kit (Thermo Fisher Scientific, Waltham, MA, USA) and from K562 leukemia cell line using the Gen Elute Mammalian Total RNA miniprep kit (Sigma-Aldrich, St. Louis, MO, USA). The high Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) was used to prepare cDNA from 1 μg of RNA. For qPCR, cDNA was amplified in a Stratagene 3000MxPro thermocycler (Agilent Technologies La Jolla, CA, USA) in triplicate with WT1, CCNA1, VEGF and the endogenous control 18s rRNA and GAPDH primer/probes (Table IIA and B) using one of 2 methods: i) Brilliant III Ultra-Fast QPCR master mix (Agilent Technologies, La Jolla, CA, USA) with Taqman Gene Expression assays (Applied Biosystems) for AML patient samples and the K562 leukemia cell line; or ii) SYBR-Green Master Mix (Agilent Technologies) with primers obtained from Integrated DNA Technologies (IDT) for the ALL patient samples. The relative quantification of WT1, CCNA1 and VEGF gene expression in the leukemia BM vs. NBM, and in the WT1-transfected vs. empty vector control-transfected K562 cells was calculated using the $2^{(-\Delta\Delta Cq)}$ method (32).

Oncomine data analysis. The cDNA microarray data submitted by Haferlach *et al* (33) in the public database (www.oncomine.org) was analyzed for WT1, CCNA1 and VEGF mRNA expression in leukemia samples relative to control samples. In the Haferlach study, diagnostic samples (n=2,096) were obtained from 750 ALL, 542 AML, 448 chronic lymphoblastic leukemia (CLL), 76 chronic myelogenous leukemia (CML), 206 leukemia precursors (LP) and 74 mononuclear cells (MNC), investigated across 11 laboratories. The following reporters were used: WT1 (206067_s_at), CCNA1 (205899_at) and VEGF (210512_s_at), and the data were grouped by leukemia types, selecting all samples in the database.

Site-directed mutagenesis. The human WT1 expression construct, pcb6+WT1, isoform A, was obtained from Dr Frank Rauscher (34). Site-directed mutagenesis was employed to reproduce a common frameshift/truncation mutation identified clinically in WT1 gene, the exon 7 frameshift (5,35,36). The 'mutant' WT1 expression plasmid was created with the QuikChange II site-directed mutagenesis kit (Agilent Technologies) using specific primers (forward, 5'TGTGCG ACGTGTGCCCTGGAGTAGCCC-3'; and reverse, 5'-GGG CTACTCCAGGGCACACGTCGCACA-3') designed using the Agilent QuikChange Primer Design Program that introduced a

Table I. Clinical characteristics of the pediatric ALL patients (n=19).

Characteristic	No.
Male	9
Female	10
Median age, years	9
Age range, years	1-16
Subtypes	
Early B cell	16
T-Cell ALL	1
T/NK ALL	1
ALL-L3	1

ALL, acute lymphocytic leukemia; T-cell ALL, T-cell acute lymphoblastic leukemia; T/NK ALL, T-cell/natural killer cell acute lymphoblastic leukemia; ALL-L3, acute lymphoblastic leukemia with an L3 morphological FAB type.

single base 'C' at position 1303-1304 (NM_024426) at exon 7. The PCR conditions were as follows: 95˚C for 30 sec followed by 18 cycles of 95˚C for 30 sec, 68˚C for 1 min and 68˚C for 18 min. The restriction enzyme, *Dpn*1, was added to digest and degrade the parental strands, and XL-1 Blue Supercompetent cells (Agilent Technologies) were then transformed with the mutant DNA. Plasmids were purified using the Qiagen plasmid kit (Qiagen, Valencia, CA, USA) and sequenced to verify correct base changes. Plasmids were used for transfection as described below.

Transfection and luciferase assays. The leukemia cell line, K562 (CCL-243; ATCC, Manassas, VA, USA), was cultured in RPMI medium with 10% fetal calf serum and antibiotics (100 IU/ml penicillin and streptomycin) in a 5% CO_2 incubator at 37˚C. The K562 cells were transfected with either the wild-type or ZF mutant human WT1 expression plasmids, or the empty vector control using Lipofectamine LTX and the LTX plus reagent (Thermo Fisher Scientific). For luciferase assays, the cells were co-transfected with the CCNA1 luciferase reporter constructs either containing the distal promoter region (-1,180/+145) or lacking it, referred to as proximal promoter (-454/+145). Where stated, the murine GFP-tagged Wt1 expression plasmids were also transfected along with the proximal or distal CCNA1 luciferase reporter. To examine the effects of mithramycin A (Sigma-Aldrich) on promoter activity, the cells were treated with 30 nM mithramycin A for 48 h following transfection. The luciferase activity of the lysates was measured on a Turner 20/20n luminometer (Turner Biosystems, Sunnyvale, CA, USA) and relative light units (RLU) normalized to protein concentrations were reported.

WT1 binding site prediction and chromatin immunoprecipitation (ChIP). A total of 3 kb of the CCNA1 promoter gene sequence was downloaded from the public database, Ensembl (http://useast.ensembl.org/) and potential WT1 binding sites (GnGGGnG; Matrix Family Library Version 9.3) were queried using MatInspector Software (https://www.genomatix.de/). The binding function of these predicted sites was tested in WT1-transfected K562 cells using ChIP assays (Millipore EZ-Magna ChIP, Temecula, CA, USA), following manufacturer's instructions. WT1-bound fragments were immunoprecipitated using a mix of the WT1 antibodies (2.5 μg each of N18 and C19; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or 1 μg mouse IgG fraction provided with the kit as a negative control. WT1 bound chromatin was amplified by end-point PCR using primers specific for each of the three predicted sites (Table IIC) and analyzed by electrophoresis in ethidium bromide-containing agarose gels. The specificity of WT1 binding was tested by the amplification of immunoprecipitated chromatin using negative control primers located approximately 1.5 kb 5' of the third WT1 site and lacking any potential WT1 binding sites.

Western blot analysis and immunofluorescence (IF). Cell lysates were harvested using RIPA lysis buffer (Millipore, CA, USA) containing protease inhibitors and EDTA (Thermo Fisher) from the K562 cells treated with 25 μM curcumin (Sigma-Aldrich) or 70 nM mithramycin A (Sigma-Aldrich). Protein concentrations were measured using Bicinchoninic acid protein assay and twenty-five micrograms of proteins were separated on a 10% separating gel by gel electrophoresis, and transferred to PVDF membrane for western blot analysis. The membranes were blocked in Phosphate Buffer Saline-Tween with 5% non-fat milk and probed overnight with anti-WT1 at a 1:1,000 dilution (#5G11A5; ab201948; Abcam, Cambridge, MA, USA), anti-CCNA1 at a 1:1,000 dilution (#B882; 556600; BD Pharmingen, San Diego, CA, USA) or anti-actin at a 1:3,000 dilution (#A00702; Genscript, Piscataway, NJ, USA) primary antibodies and antimouse IgG-HRP at a 1:10,000 dilution (#HAF007; R&D Biosystems, Minneapolis, MN, USA) as secondary antibodies. Proteins were visualized by chemiluminescence detection with Fuji LAS 3000 (GE Healthcare, Piscataway, NJ, USA). The quantification of the bands was performed using ImageJ Version 1.51p.

The K562 cells treated with 25 μM curcumin or 70 nM mithramycin A for 48 h were fixed onto poly-lysine coated coverslips to stain for IF analysis. The coverslips were incubated with anti-CCNA1 (B882; BD Pharmingen) primary at 1:50 dilution and Cy3-fluorophore tagged antibodies at 1:1,000 (#115-165-003; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and stained with DAPI (Vector Laboratories, Burlingame, CA, USA). Images were acquired with a 60X objective lens in bright field and fluorescence at emission maximum of 461 nm (blue) for DAPI-stained nuclei and 569 nm (red) for Cy3-tagged antibodies and visualized on an Olympus IX81 microscope (Olympus, Tokyo, Japan). The average staining intensity was quantified using ImageJ software and the intensity in the treated cells was plotted relative to the control cells.

MTT and trypan blue dye exclusion assays. The effects of mithramycin A on K562 cell viability and proliferation were examined using 3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (Trevigen, Gaithersburg, MD, USA) and trypan blue dye (Sigma-Aldrich) exclusion assays. Briefly $2.5x10^4$ K562 cells were seeded into 96-well plates in triplicate

Table II. Primer sequences.

A, Primers used for qPCR (SYBR-Green)[a]

Functional class	Gene	Forward primer	Reverse primer
Housekeeping	GAPDH	5'-CCATCACCATCTTCCAGGAG-3'	5'-GGATGATGTTCTGGAGAGCC-3'
Transcription factor	WT1	5'-GAGAGCCAGCCCGCTATTC-3'	5'-CATGGGATCCTCATGCTTG-3'
Growth control	CCNA1	5'-AAGAAGCAGCCAGACATCAC-3'	5'-GCAGTTTCCCTCTCAGAACA-3'
Growth control	VEGF	5'-CGAAACCATGAACTTTCTGC-3'	5'-CCTCAGTGGGCACACACTCC-3'

B, Primers used for qPCR (TaqMan)[b]

Housekeeping	18s	Hs99999901_S1
Transcription factor	WT1	Hs00240913_m1
Growth control	CCNA1	Hs00171105_ m1
Growth control	VEGF	Hs00900057_m1

C, Primers used for ChIP

	Forward primer	Reverse primer
CCNA1 (site 1)	5'-GGAAACAGTCCCTTCCAAA-3'	5'-TGAATCGGCCAATCAGC-3'
CCNA1 (site 2)	5'-ACCTGG CTT GTCAGT CACCTAT-3'	5'-GTCCCCACGAGAGGTCTTC-3'
CCNA1 (site 3)	5'-ACAGGTTCCACGAACAAACACTGC-3'	5'-AGGAGCATTTCTGTCCTCTTG CCT-3'
Negative control ChIP[c]	5'-TCAGGAGATCGAGACCATCCTGTGTA-3'	5'-CAACCTGGCAGTGAATGCTCAACT-3'

[a]Primers used for SYBR-Green qPCR for GAPDH, WT1 and VEGF were obtained from IDT DNA technologies and CCNA1 was obtained from realtimeprimers.com. [b]Primers used for TaqMan qPCR were obtained from ABI. [c]Negative control ChIP-negative control region primers targeting the 5' promoter region lacking any identified WT1 binding sites.

and treated with either the diluent control (DMSO), or 50, 70 and 100 nM mithramycin A for 48 h. The cells were then incubated with MTT reagent as per the manufacturer's recommendations and the absorbance was measured with BioTekCytation5 (BioTek Instruments, Winooski, VT, USA) plate reader at 630 nm for substrate and 570 nm for background normalization. Average normalized substrate absorbance values were plotted as percentage of metabolically active mithramycin A-treated cells relative to the DMSO-treated control cells. The cells were also stained with trypan blue (Sigma-Aldrich) and counted on a hemocytometer (Sigma-Aldrich), and the average numbers of viable cells remaining after 48 h of treatment were reported.

Statistical analysis. Statistical significance was determined by the non-parametric Mann-Whitney U test for patient samples using GraphPad Prism software version 8.0.0. Statistical significance for experiments using the K562 cells was determined by ordinary one-way ANOVA, followed by Dunnett's multiple comparison test in experiments involving >2 groups and the Student's t-test in experiments involving only 2 groups. Significance was determined at $P<0.05$.

Results

WT1 and CCNA1 mRNA levels are elevated in pediatric AML-M3 BM samples. The expression of WT1, CCNA1 and VEGF was evaluated by RT-qPCR analysis of 19 pediatric ALL and 10 pediatric AML-M3 BM samples (Fig. 1A). The low expression of WT1, CCNA1 and VEGF was observed in the pediatric ALL samples, with the median WT1 and CCNA1 expression being similar to that in NBM, while the median VEGF expression was significantly lower (Fig. 1A, left panel). In the ALL samples, 6 had elevated (>2-fold above NBM) WT1 levels and 5 had elevated CCNA1 levels (data not shown), but all had VEGF levels lower than NBM. In the AML-M3 samples, the median WT1 and CCNA1 expression was higher than that in NBM, while the VEGF levels were lower (Fig. 1A, right panel). Thus, across both the ALL and AML-M3 sample sets examined, the VEGF levels were consistently lower than those in NBM, while the WT1 and CCNA1 expression levels varied (in ALL samples they were close to those in NBM, while in the AML-M3 samples, WT1 and CCNA1 expression was elevated above than that in NBM).

Diverse expression patterns were also observed in an analysis of an independent microarray dataset of leukemia samples from Haferlach *et al* (33) from the Oncomine database (Fig. 1B). The expression patterns observed in the various leukemia types were in general agreement with our RT-qPCR data for ALL and AML samples. The median WT1 and CCNA1 expression in the AML samples was greater than that in the MNC controls and was generally higher than that in the ALL samples. The expression of CCNA1 was lower in the ALL samples compared

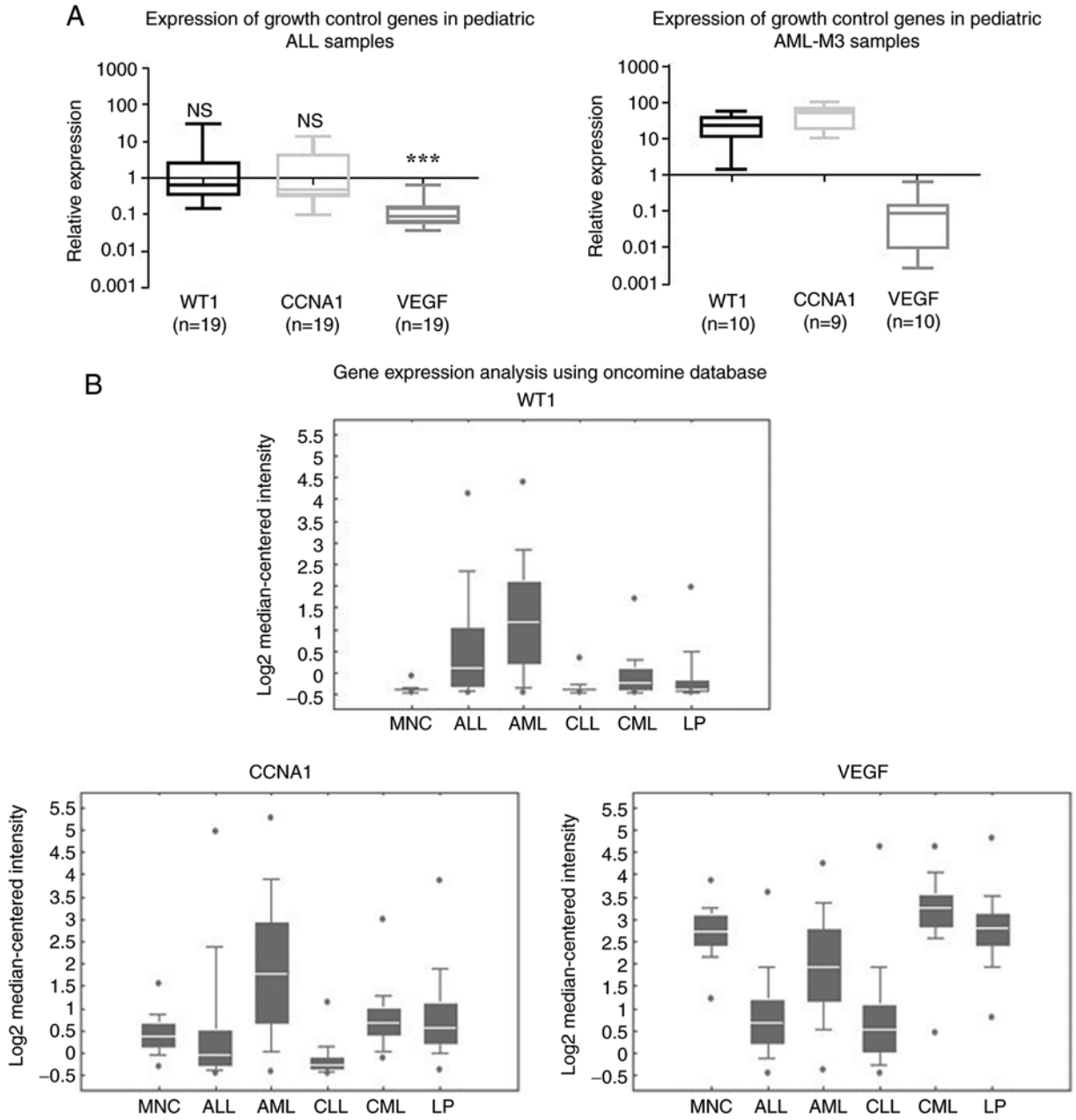

Figure 1. WT1 and CCNA1 expression was higher in acute leukemia than in normal BM samples. (A) RT-qPCR analysis of WT1, CCNA1 and VEGF expression shown as box plots in $\log_{10}$ scale demonstrating the fold change of expression in leukemia BM relative to NBM (represented by the solid line at 1.0). The median WT1 and CCNA1 expression in ALL did not vary significantly between the NBM and leukemia samples (NS; P=0.4425 and P=0.8149), while median VEGF expression was significantly lower in the leukemia group than in NBM (***P<0.001), as determined by the Mann-Whitney U test. Median WT1 and CCNA1 expression was higher in AML-M3 BM samples than in NBM, while VEGF levels were lower. (B) Oncomine analysis from microarray gene expression data collected by Haferlach *et al* (33) showing that WT1 and CCNA1 expression was higher in AML than in ALL or controls, while VEGF was lower in ALL and AML compared to the controls. WT1, Wilms tumor 1; CCNA1, cyclin A1; MNC, mononuclear cells; ALL, acute lymphocytic leukemia; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; LP, leukemia precursor; NBM, non-leukemic bone marrow.

to the controls, while the VEGF levels were lower than those in the controls in both the ALL and AML samples. Overall, these analyses demonstrated that WT1 and CCNA1 expression varied between the types of leukemia, with a higher expression being more common in AML than ALL, consistent with our analysis of pediatric leukemia samples.

Overexpression of wild-type, but not ZF mutated WT1 increases CCNA1 expression in K562 cells. Exon 7 is a hotspot area for WT1 mutations in leukemia, and the predominant type of mutation identified in this region is a frameshift caused by either insertion or deletion of one or more base pairs (5,35,36). Sequence analysis of our expression constructs comparing wild-type and mutant WT1 confirmed the insertion of a single base 'C' at exon 7 resulting in a premature stop codon and the truncation of exon 7-10 (ZF domain) at the C-terminus of WT1 protein (Fig. 2A). The transfection of WT1 plasmids into the K562 cells resulted in the expression of either the full-length wild-type (52 kDa) or the truncated mutant WT1 protein (35 kDa). A comparison of the effects of the transfection of wild-type or mutant WT1 constructs on the CCNA1 and VEGF mRNA levels in K562 cells was evaluated by RT-qPCR analysis

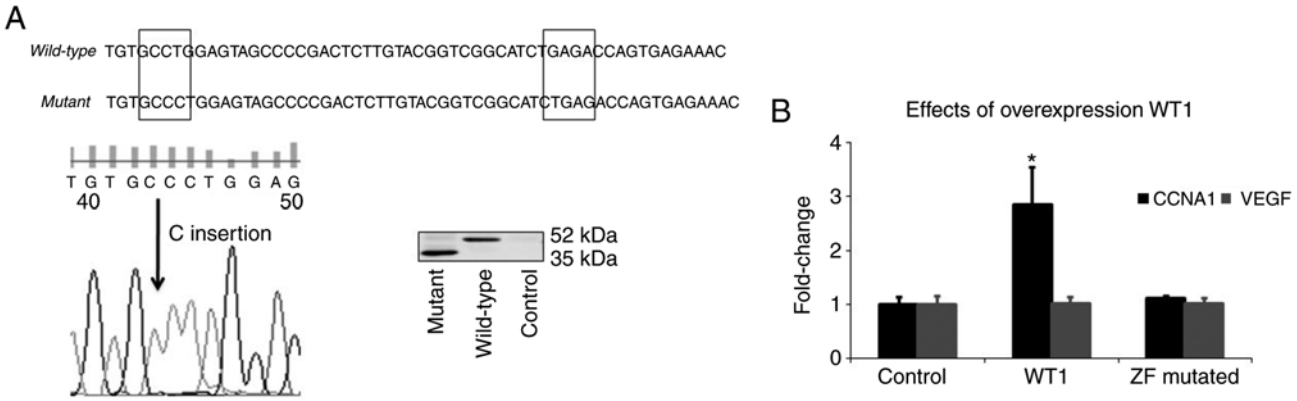

Figure 2. Wild-type WT1, but not a ZF-mutated WT1, upregulates CCNA1 mRNA in K562 cells. (A) A naturally occurring mutation in exon 7 (encoding ZF-1) of WT1 gene truncates ZF domain of WT1 protein. Construction of ZF truncation mutant, confirmation of frameshift mutation at 1,340 bp within the zinc finger 1 domain (exon 7), and production of the resultant ZF mutant truncated WT1 protein are described in methods. Sequence analysis comparing wild-type and mutant WT1 confirmed the insertion of a single base 'C' at exon 7 by site-directed mutagenesis (the region of insertion shown in box on the left) which resulted in a premature stop codon (box on the right), and truncation of exon 7-10 at the C-terminal end of WT1 protein. Following the transfection of WT1 plasmids into K562 cells, western blot analysis (right panel) revealed the expression of both wild type (52 kDa) and mutant (35 kDa) WT1 protein. (B) CCNA1 expression was upregulated approximately 2.5-fold in K562 cells transfected with wild-type WT1 construct relative to the cb6+ empty vector control, but no enhancement in CCNA1 mRNA expression was observed in ZF mutant WT1-transfected K562 cells. VEGF levels remained unaltered by WT1 transfection. Statistical significance was determined by one-way ANOVA (*P<0.05). WT1, Wilms tumor 1; CCNA1, cyclin A1; VEGF, vascular endothelial growth factor.

(Fig. 2B). The transfection of wild-type WT1 increased the mRNA levels of CCNA1 (relative to the empty vector), but not those of VEGF, and the ZF mutant WT1 did not upregulate the expression of any gene targets tested. These results suggest that CCNA1 is a potential WT1 target gene. In addition, since the mutant plasmid lacking the ZF domain failed to enhance expression, an intact DNA binding domain is required for the WT1 mediated upregulation of CCNA1.

Treatment of K562 cells with curcumin decreases the WT1 and CCNA1 protein levels. Curcumin has been shown to suppress the WT1 mRNA and protein levels in K562 cells (37). In addition to the curcumin-induced decrease in the WT1 protein levels, we observed a reduction in the CCNA1 protein levels in the K562 cells. Curcumin at the concentration of 25 μM decreased WT1 and CCNA1 protein expression in the K562 cells, as demonstrated by western blot analysis (Fig. 3A). The suppression of CCNA1 protein expression was also confirmed by IF, showing that treatment with 25 μM curcumin decreased the intensity of CCNA1 staining (Fig. 3B and C). While these results provide evidence of the coordinate downregulation of both CCNA1 and WT1 expression by curcumin treatment, they are also consistent with the lack of CCNA1 mRNA expression observed in the absence of WT1 expression (or when WT1 mutant constructs are expressed in K562 cells) shown in Fig. 2B.

CCNA1 promoter region contains functional WT1 binding sites. Potential WT1 binding sites were queried using MatInspector Software to analyze 3 kb of the CCNA1 promoter gene sequence obtained from the public database, Ensembl, and 3 potential WT1 binding sites were identified at -1,779, -1,057 and -499 bp upstream of the transcription start site (Fig. 4A). To examine WT1 binding at these sites *in vivo*, ChIP assays were carried out and WT1 bound chromatin was amplified by end-point and qPCR using primers (Table IIC) specific for the 3 predicted sites. WT1 binding to the CCNA1 promoter at sites 3 and 2 was revealed by the modest enrichment of DNA precipitated by WT1 antibody, compared to IgG, as shown by gel electrophoresis (Fig. 4B) and confirmed by qPCR (shown as bar chart in Fig. 4B bottom panel). By contrast, no WT1 binding was observed at site 1, as evidenced by the absence of amplification of that region. The specificity of WT1 binding was also indicated by the absence of amplification of a negative control region located approximately 1.5 kb upstream of the third WT1 site. Taken together, these results suggest that the specific *in vivo* binding of WT1 to the CCNA1 promoter in chromatin of the K562 cells mediates the transcriptional regulation of CCNA1 through binding to its promoter.

Activation of the CCNA1 promoter by WT1 requires ZF domain, but not canonical WT1 binding sites. The co-transfection of distal CCNA1 promoter plasmid (Fig. 4C) into K562 cells with one of the expression constructs described in Fig. 2A revealed that the wild-type WT1 increased the distal CCNA1 promoter activity by >2.5-fold relative to the empty vector control, but the mutant lacking the ZF domain did not enhance CCNA1 promoter activity (Fig. 4D). Surprisingly, although no WT1 binding sites were identified in the GC-rich proximal region of the CCNA1 promoter (Fig. 4C), WT1 significantly elevated proximal promoter (-454/+145) activity (Fig. 4E). The upregulated distal and proximal promoter activity was confirmed by transfection of the K562 cells with the murine GFP-tagged Wt1 construct (Fig. 4F and G). These results suggest that the WT1 ZF domain is important for the transcriptional upregulation of the CCNA1 promoter, and that WT1 activates the promoter through both canonical and non-canonical sites.

Mithramycin A decreases CCNA1 expression and the viability of K562 cells. To confirm the importance of the GC-rich region for CCNA1 promoter activity, the K562 cells were treated with mithramycin A to block GC binding. Mithramycin A at even low concentrations (30 nM) decreased distal promoter (containing WT1 binding site #2) activity by 70% and

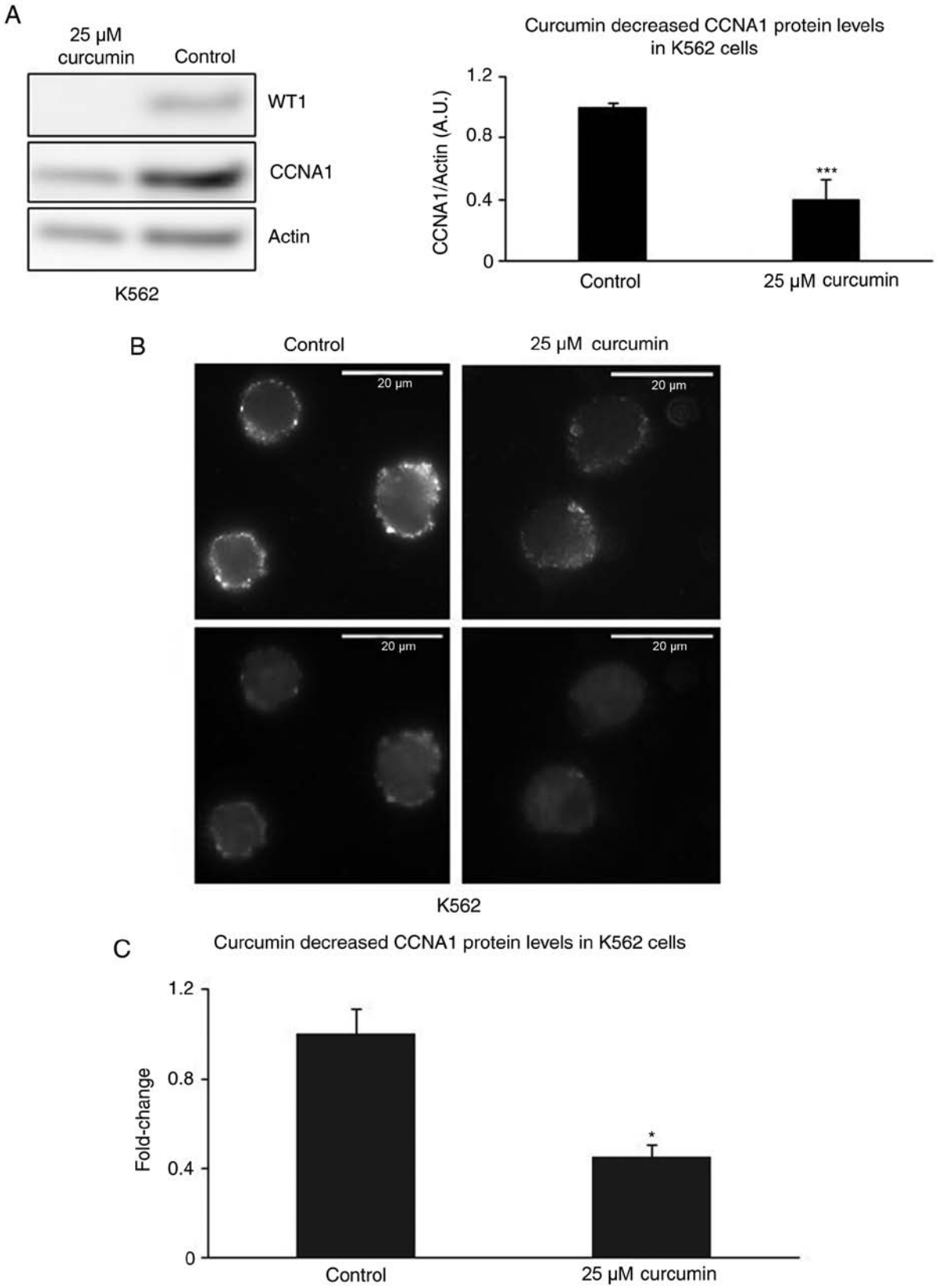

Figure 3. Curcumin decreases WT1 and CCNA1 protein levels in K562 cells. (A) Protein lysates collected from K562 cells treated with 25 μM curcumin for 48 h exhibited reduced levels of WT1 and CCNA1 protein expression compared to DMSO-treated control cells. Quantification of chemiluminescence was carried using ImageJ and CCNA1 levels normalized to actin (means ± SD) are shown. Significance was determined using a Student's t-test at ***P<0.001. (B) CCNA1 protein expression in curcumin-treated K562 cells measured by immunofluorescence was also reduced compared to DMSO-treated control cells. Images were acquired using a 60X objective lens at an emission maximum of 461 nm (blue) for nuclei counterstained with DAPI and 569 nm (red) for CCNA1 counterstained with Cy3. Shown are representative images of CCNA1 expression in K562 cells (top panel) and composite images with stained nuclei (bottom panel) following DMSO (left panel) or 25 μM curcumin (right panel) treatments. Scale bars, 20 μm. (C) Curcumin decreased the intensity of CCNA1 protein staining by 55% compared to DMSO-treated cells as quantified using ImageJ. The average integrated density in treated cells was plotted relative to the control cells and expressed as fold change relative to the control. Error bars represent standard deviation. Significance was determined using a Student's t-test at *P<0.05. WT1, Wilms tumor 1; CCNA1, cyclin A1.

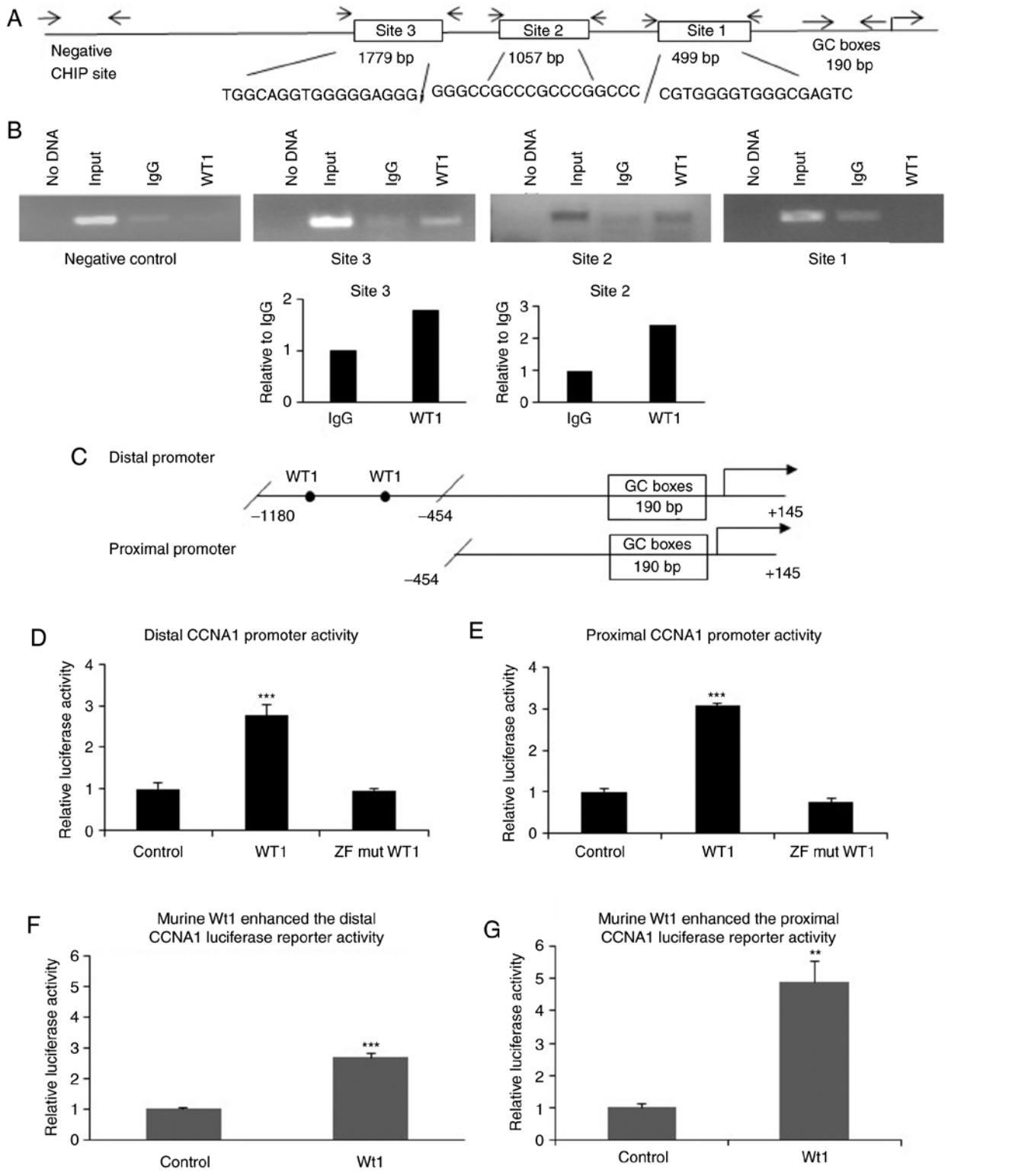

Figure 4. WT1 binding is observed at the CCNA1 promoter, and WT1 overexpression upregulates CCNA1 promoter activity. (A) Three potential WT1 binding sites in the CCNA1 promoter were identified using MatInspector software. Arrowheads indicate primer locations used to PCR-amplify each individual binding site (primer sequences shown in Table IIC). (B) ChIP assay demonstrated chromatin binding by WT1. Purified DNA was PCR amplified at sites 1, 2 and 3, and a negative control site in the CCNA1 promoter, then analyzed by gel electrophoresis. WT1 bound *in vivo* to chromatin at the sites -1,779 (site 3) and -1,057 (site 2), but not to the site -499 (site 1) or to the negative control site lacking WT1 sites (specificity control). The lanes from left to right are as follows: lane 1, PCR control (no DNA); lane 2, input DNA control; lane 3, IgG (antibody control); lane 4, WT1 antibody precipitated chromatin. Binding at sites 2 and 3 was also verified by qPCR amplification and shown as fold enrichment relative to IgG (bottom panel). (C) Schematic diagram of the distal (-1,180/+145) and proximal (-454/+145) CCNA1 promoter with filled circles indicating WT1 binding sites. (D) Distal and (E) proximal CCNA1 promoter constructs were co-transfected with the human WT1 expression plasmids (wild-type and ZF mutant) in K562 cells. Shown is the normalized luciferase activity of CCNA1 promoters in WT1-transfected cells relative to the empty vector control. Wild-type WT1 upregulated the distal (-1,180/+145) and the proximal (-454/+145) CCNA1 promoters, while the ZF mutated construct did not increase promoter activity. Significance was determined at $^{***}P<0.001$ using one-way ANOVA. Results of transfection of human WT1 expression plasmids were confirmed with murine Wt1 plasmids. Luciferase activity in (F) distal and (G) proximal CCNA1 promoter co-transfected with the murine Wt1 expressing plasmid demonstrated upregulated activity compared to empty vector control. Significance was determined at $^{**}P<0.01$ and $^{***}P<0.001$ using Students t-test. WT1, Wilms tumor 1; CCNA1, cyclin A1.

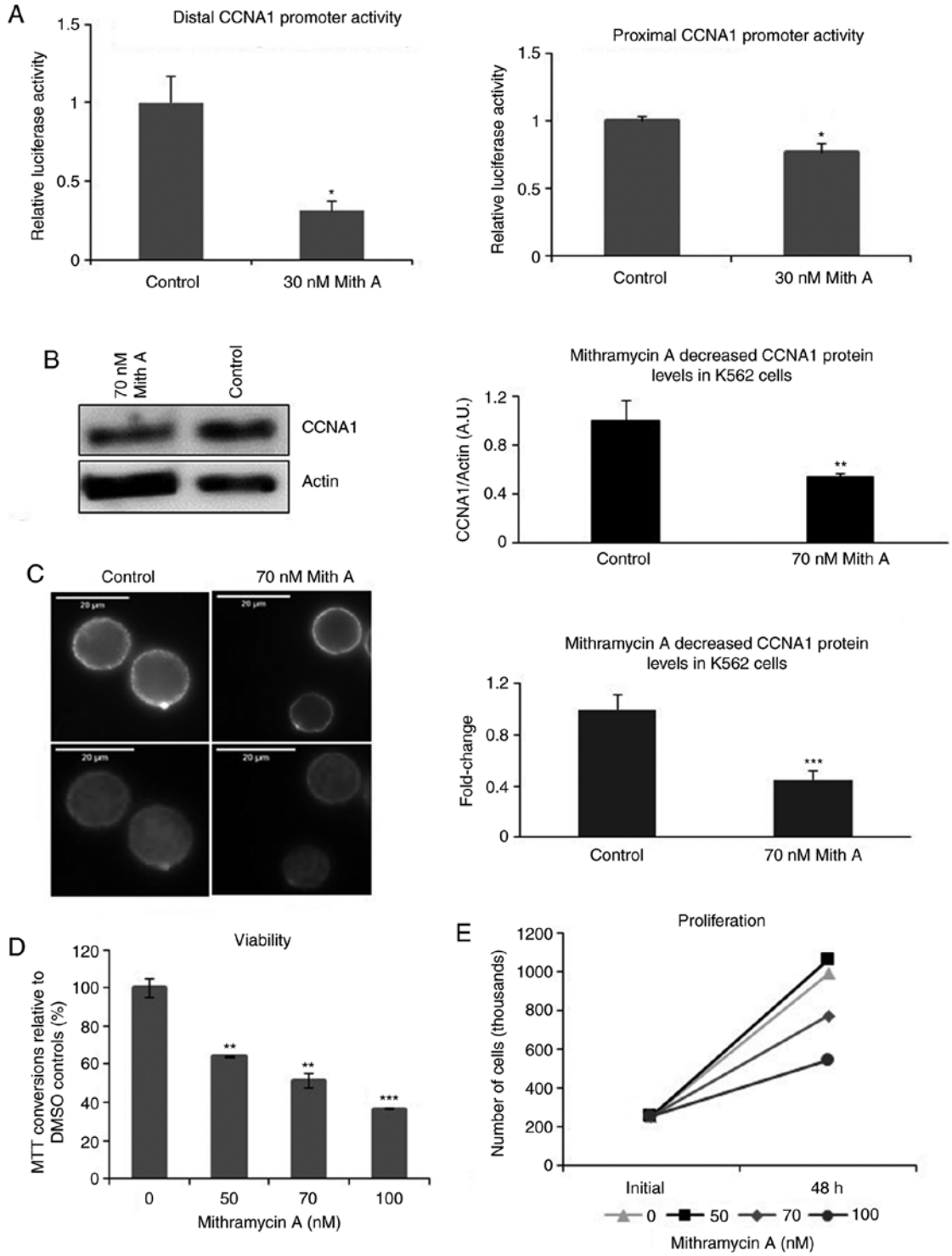

Figure 5. Mithramycin A treatment decreases CCNA1 promoter activity, protein expression, and the proliferation of K562 cells. (A) K562 cells were transfected with either the distal or proximal CCNA1 promoter constructs and luciferase activity measured following 48 h of treatment with 30 nM mithramycin A or DMSO as the diluent control. Mithramycin A reduced the activity of the distal CCNA1 promoter (-1,180/+145) containing the WT1 binding site #2 and had a modest but reproducible effect on the proximal promoter (-454/+145) relative to DMSO controls. Significance was determined using a Student's t-test ($^{*}P<0.05$). (B) Cytoplasmic protein lysates from K562 cells treated for 48 h with either 70 nM mithramycin A or DMSO were analyzed by western blot analysis, and the mithramycin A-treated cells exhibited a decrease in CCNA1 protein expression as compared to the DMSO controls. Actin was used as the loading control and protein was quantified using ImageJ (means ± SD). Significance was determined using a Student's t-test ($^{**}P<0.01$). (C) Mithramycin A treatment decreased the intensity of CCNA1 staining measured by immunofluorescence (as described in Fig. 3) when compared to the DMSO-treated control cells. The top panel shows CCNA1 expression and the lower panel shows composite images with stained nuclei. Scale bars, 20 μm. Images were quantified using ImageJ and shown as the fold change in integrated density relative to the DMSO-treated control. Error bars represent standard deviation. Significance was determined using a Student's t-test at $^{***}P<0.001$. (D and E) Treatment of the K562 cells with 0, 50, 70 and 100 nM mithramycin A decreased their metabolic activity and viability as measured by MTT and Trypan blue exclusion assays, respectively. (D) Shown are average normalized substrate absorbance values plotted as percentage of metabolically active mithramycin A-treated cells relative to the DMSO-treated control cells, as determined by MTT assay. Significance was determined by one-way ANOVA and significance was determined at $^{**}P<0.01$ and $^{***}P<0.001$. (E) Viable cells were identified by trypan blue exclusion assay and counted on a hemocytometer. Numbers of viable cells remaining after 48 h of treatment are reported. Mith A, mithramycin A; WT1, Wilms tumor 1; CCNA1, cyclin A1.

proximal promoter (containing no WT1 binding site) by 23% in the treated K562 cells compared to the DMSO-treated cells (Fig. 5A). This dampening of CCNA1 expression resulted in a 2-fold reduction in the CCNA1 protein levels in the 70 nM mithramycin A-treated cells examined by western blot analysis (Fig. 5B) and confirmed by IF (Fig. 5C). The intensity of CCNA1 staining was decreased >2-fold in the mithramycin A-treated K562 cells (Fig. 5C, right panel), compared to that of the DMSO-treated cells (Fig. 5C, left panel).

The effects of the mithramycin A-induced decrease in the CCNA1 protein levels on the proliferation of K562 cells were examined by MTT and trypan blue exclusion assays. The K562 cells treated with increasing concentrations of mithramycin A exhibited a decrease in viability after 48 h (Fig. 5D), but not at 24 h (data not shown), as assessed by MTT assay. To determine whether mithramycin A causes cell death or only decreases the proliferative potential, viable cells were identified by trypan blue dye exclusion and counted on a hemocytometer. The number of viable cells after 48 h of mithramycin A treatment was lower compared to that observed with DMSO treatment, but was nevertheless still above the initial numbers seeded, even at the highest concentrations (Fig. 5E), suggesting mithramycin A was not cytotoxic, but rather anti-proliferative.

Discussion

Clinical studies on leukemia patient samples have linked an increased WT1 expression to an increased risk of relapse (38) and an increased CCNA1 expression to poor clinical responses (6,11). The importance of CCNA1 expression is still under study; however, recently, CCNA1 was described to regulate pathways important for the homing of HSPCs in their BM niche, with clear implications for the development of both normal hematopoietic and leukemia stem cells (20).

In this study, the comparison of WT1 and CCNA1 mRNA in pediatric ALL and AML-M3 exhibited the consistent, elevated expression of WT1 and CCNA1 in the AML-M3 relative to the non-leukemia BM samples. By contrast, their expression in the pediatric ALL samples varied widely and was generally lower than that in the AML-M3 samples. Concordant with the RT-qPCR results, Oncomine analysis of the microarray data submitted by Haferlach *et al* (33) revealed a higher WT1 and CCNA1 expression in AMLs compared to ALLs. The significance of the wide range of WT1 expression in pediatric ALL, which is the predominant pediatric leukemia type (39), is unclear, but has also been reported by others (40). Previously, other studies have also reported WT1 and CCNA1 levels to be elevated in myeloid leukemia, particularly in the AML-M3 subtype (41,42), more than in lymphocytic leukemia (42-44).

The relevance of elevated WT1 in acute leukemia is most likely linked to its role as a transcription factor (reviewed in ref. 45). In this study, since WT1 and CCNA1 were coordinately expressed in our pediatric AML-M3 populations, we examined the effects of WT1 on CCNA1 mRNA expression. WT1 overexpression in the myelogenous leukemia cell line, K562, increased CCNA1 mRNA expression. This is the first report demonstrating the WT1 regulation of CCNA1 in cancer cells. While our results indicate that WT1 transcriptionally enhances CCNA1, there may also be a negative feedback loop whereby elevated CCNA1 somehow leads to suppression of WT1 expression and a redistribution of the normal isoform ratios (18). Herein we demonstrated that WT1 activated the distal CCNA1 promoter containing 2 potential WT1 binding sites. This activation required DNA binding by WT1, as the mutant WT1 (lacking the ZF DNA binding domain) failed to activate. Conversely, treatment of the K562 cells with curcumin, known to suppress WT1 mRNA and protein levels in various cancer cell lines (37,46,47), was found to be associated with reduced CCNA1 protein levels. Since curcumin is a multi-targeted drug [reviewed in (48)], this novel discovery of the effects of curcumin on CCNA1 expression and the association with decreased WT1 protein levels in K562 cells suggests a plausible link between WT1 and CCNA1. However, evidence of a direct link indicating the necessity of WT1 for CCNA1 regulation awaits the gene-specific silencing of WT1 expression.

WT1 was observed to bind to chromatin in the distal CCNA1 promoter region and WT1 expression upregulated the distal CCNA1 (-1,180/+145) promoter activity. Surprisingly, WT1 also activated the proximal CCNA1 (-454/+145) promoter containing no canonical WT1 binding sites but including the GC-rich core. The core CCNA1 promoter region (-190/+145) contained 4 major GC boxes that others have shown to be critical for promoter activity, as the mutation of these GC-boxes decreases promoter activity (49). The ZF transcription factor, SP1 (50), binds at these GC-boxes and plays a pivotal role in regulating this promoter region and CCNA1 expression (49). Other ZF transcription factors, such as EGR1 and WT1, also bind at GC-rich regions, such as those located in the promoter of PDGF A-chain gene (51). In fact, binding sites for WT1 (-GNGNGGGNG-), SP1 (-GGGCGG-) and EGR1 (GCG(T/G)GGGCG) often overlap (52) and these ZF factors can displace one another, competing for binding at the same sites (53). Hence, we hypothesized that WT1 binds at these GC-rich sites and activates the CCNA1 core promoter region. However, PCR amplification of chromatin in this core region failed, due to the high GC content, and thus direct evidence of WT1 binding at the core promoter awaits ChIP analysis. Nevertheless, our observation that WT1 transfection upregulated activity of the proximal CCNA1 promoter lacking canonical WT1 binding sites, but enriched for GC boxes, suggests either direct binding by WT1 at the GC sites, or indirect binding by a complex containing WT1 and a GC-box binding factor, such as SP1.

To confirm the importance of the GC-rich core region of the CCNA1 promoter, K562 cells were treated with mithramycin A, which binds to the minor groove of DNA at GC-rich regions and competitively inhibits binding of ZF motifs (54). Mithramycin A treatment decreased both the distal and proximal CCNA1 promoter activity and endogenous CCNA1 protein expression in the K562 cells. Importantly, this reduction was associated with a decreased cell viability and the decreased proliferation of K562 cells.

In addition to WT1 and CCNA1, VEGF gene expression was also quantified in our analysis of pediatric leukemias. However, unlike WT1 and CCNA1, the VEGF levels were lower in the AML-M3 than in the NBM control samples. In agreement with our findings on mRNA expression, others have shown that the median VEGF protein levels in serum samples from diagnostic pediatric ALLs were also lower compared to normal controls (55,56). Indeed, VEGF mRNA levels at initial diagnosis in pediatric ALLs have been shown to be lower than

those at relapse, and in serial samples, VEGF levels have been shown to increase at relapse (57). This is consistent with our study population of pediatric patients with low VEGF levels in BM samples collected at first diagnosis. Our RT-qPCR results were further supported by the analysis of an independent microarray data set of leukemia samples from the Oncomine database that showed VEGF levels in ALL and AML samples were significantly lower than in normal controls (33).

Overall, the generally low expression of CCNA1 and WT1 in pediatric ALL would suggest that they are not common in pediatric ALL. However, in those patients with elevated CCNA1 and WT1 levels, they may indicate a cause for concern, as elevated levels have been associated with poor outcomes (11). This study provides evidence of the ability of WT1 protein to bind and regulate expression of the CCNA1 gene promoter, supporting a mechanistic relationship. Given the potential for WT1 to alter the homing of HSPCs by modulating CCNA1 levels, this would suggest the utility of monitoring both WT1 and CCNA1 levels in patients at risk of relapse. If WT1 and CCNA1 expression were used to monitor for evidence of MRD in pediatric population, they could provide early evidence of stem cell transplantation success. Additional studies are required; the identification of genes that drive relapse would lead to an improved prognosis and ultimately, to the development of novel effective therapies.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article or are available from the corresponding author on reasonable request. Constructs and data are available upon request.

Authors' contributions

SJK, SP and GCF conceived and designed the study. SP, MM and NG performed the experiments and collected the data. SP and GCF analyzed the data. SP, SJK and GCF wrote the preliminary draft of the manuscript. All authors have proofread and edited the manuscript and all authors have read and approved the final manuscript.

Ethics approval and consent to participate

Diagnostic BM aspirates from 20 pediatric ALL patients were obtained in accordance with Akron Children's Hospital IRB guidelines (including patient consent to participate, and evaluation of ethical considerations). Samples in excess of that needed for diagnosis were provided for research purposes. Informed consent was obtained from all parents/legal guardians of minor children for use of diagnostic leukemic bone marrow specimens for the research. Non-leukemia specimens were obtained as discarded sample from bone marrow aspirations performed on patients for various clinical indications (e.g., staging of solid tumors, evaluation of non-leukemic hematologic conditions such as ITP, etc.). An additional 10 RNA samples were obtained from the Children's Oncology Group, also obtained through IRB approval. No data about patient characteristics were available for this cohort.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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