Survivin is a novel transcription regulator of KIT and is downregulated by miRNA-494 in gastrointestinal stromal tumors

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Gain-of-function mutations of KIT are pathognomonic in sporadic gastrointestinal stromal tumors (GISTs). Several microRNAs have been shown to be dysregulated in GISTs and impact KIT expression. Little is known though on KIT-independent targets of KIT-regulating mRNAs. We sought to investigate how miR-494 inhibits GIST proliferation and to identify novel target gene. We used microarray-based gene expression analyses to identify pathways and target genes affected by miR-494. The expressional relationship between survivin and miR-494 was determined in 35 GIST tissues. Cell proliferation assay, FACS analysis, colony formation assay, promoter assays and chromatin immunoprecipitation (ChiP) were performed to clarify the roles of survivin in GIST progression. Gene expression microarray analysis revealed that miR-494 inhibited GISTs by affecting multiple genes in the cell cycle pathway. *Survivin (BIRC5)* was a key target of miR-494, and its expression showed an inverse correlation with miR-494 expression in 35 GIST tissues (Pearson's correlation coefficient, $r = -0.418$, $p = 0.012$). Downregulation of survivin inhibited proliferation and colony formation, and resulted in cell cycle alteration. Induced survivin overexpression relieved miR-494-mediated inhibition of GIST progression. Targeting PI3K effectively suppressed proliferation of GISTs with downregulation of survivin. Survivin also regulated *KIT* expression at the transcription level. Immunohistochemical analysis using 113 GISTs revealed that survivin expression was significantly correlated with overall survival of GIST patients ($p = 0.004$). Our findings indicated that miR-494 synergistically suppressed GISTs by concomitantly targeting survivin and KIT.

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Introduction

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of the gastrointestinal tract. Mutually exclusive single-gene mutations in *KIT* (75%) and *PDGFRA* (10%) are thought to be the initiating genomic event in the oncogenic progression of sporadic GISTs.[1-4] By contrast, GISTs arising in the absence of mutations in *KIT* or *PDGFRA* are attributed to functional defects in the mitochondrial succinate dehydrogenase (SDH) complex, which is composed of subunits SDHA, SDHB, SDHC and SDHD.[5] These SDH-deficient GISTs harbor mutations in one of the four SDH isoforms.[5] KIT remains the major oncogenic driver even in patients with widespread metastatic disease, which explains the success of specific KIT-inhibitors. Imatinib blocks autophosphorylation of KIT as well as KIT-dependent signaling such as RAS/RAF/MAPK and PI3K pathways, which subsequently inhibits proliferation or induces apoptosis.[6] While imatinib shows long lasting remissions, most patients eventually progress with only a minority of patients permanently responding to treatment.[7] Notably, KIT remains the major driver of the disease even after progression on imatinib as patients develop secondary resistance mutations in the kinase domain of KIT.[8] As many patients develop multiple different resistant clones at the same time, effective pharmacological inhibition remains an obstacle in clinical

What's new?

Gain-of-function mutations of KIT are pathognomonic in sporadic gastrointestinal stromal tumors (GISTs). Several microRNAs have also been shown to be dysregulated in GISTs and impact KIT expression. However, still little is known on KIT-independent targets of KIT-regulating mRNAs. Here, the authors report that miR-494 induces alteration of the cell cycle pathways in GISTs by downregulating survivin, which functions as a transcription factor of *KIT*, accompanying effective blockade of the PI3K pathway that both KIT and survivin belong to. Based on these findings, miR-494 may play important roles during GIST tumorigenesis and be used as a potent agent to treat GISTs.

practice.[9] Thus, current salvage therapies exhibit only moderate disease control rates and patients failing imatinib are faced with a poor outcome.[10,11] Novel approaches that inhibit *KIT*—regardless of secondary mutations—as well as the identification of additional targetable driver genes in GISTs are required.

Although a wide range of therapies that inhibit *KIT* at the protein level are now available, therapeutic approaches enabling mRNA-level regulation of *KIT* expression have been rarely reported.[10,12] Loss of chromosome 14q is a frequent cytogenetic event (80% in gastric GISTs).[13] Interestingly, a cluster of forty-seven miRNAs is located on 14q32.2 and 32.31 and miRNAs in this region are frequently downregulated or silenced in several types of cancers, suggesting a tumor-suppressor role for the miRNAs in this cluster.[14,15] Only few functional studies have been performed to elucidate the role of these miRNAs in GISTs. Of note, microRNAs that selectively regulate gene expression have been shown as efficient KIT suppressors.[16] MiR-17/20a, miR-193a, miR-218, miR-221, miR-222 and miR-494 target KIT mRNAs; their expression inversely correlates with the expression of KIT in leukemia and GISTs.[17–21] MiR-494 downregulates KIT expression by binding two different sites in the 3′-UTR of KIT mRNAs in GISTs, inhibit proliferation, and eventually induce GIST cell apoptosis.[20] Low expression of miR-494 that occurs with the frequent loss of the 14q chromosome (approximately 70% of GISTs) may therefore contribute to high expression levels of KIT and therefore contribute to oncogenic dependency on KIT as well as GISTs tumorigenesis.[16] A single microRNA theoretically binds to approximately 100 target genes, and some target genes show reduced microRNA-mediated protein output.[22,23] Only a limited number of the target genes form a gene set that is functionally sensitive to a specific miRNA.[22,24] Many target genes of miR-494 (e.g., *PTEN*, *CDC20* and *FGFR2*) have been identified in cholangiocarcinoma, glioblastoma and ovarian cancers.[25–27] However, *KIT* is the only critical target gene of miR-494 that has been found in GISTs.[20] A better understanding of target genes of, and pathways regulated by, miR-494 in these tumors may therefore pave the way to novel targets that maintain the oncogenic phenotype in GISTs.

We used a microarray approach to determine mechanisms of miR-494-mediated inhibition of GISTs and found that the cell cycle pathway was the most significantly affected by miR-494. We further found that *survivin* (encoded by *BIRC5*)

was directly targeted by miR-494, and was variably expressed in most GIST tissues. Our findings demonstrated that survivin had key roles in PI3K pathway dependent GIST progression and transcriptional regulation of *KIT* expression. Survivin expression also had a significant relationship with some important clinicopathological factors in a cohort of GIST patients. Taken together, these findings revealed novel functions of miR-494 and provide a therapeutic rationale for its application to GIST treatment.

Material and Methods

Cell lines and culture, dual luciferase assay, quantitative (q)PCR, western blotting and immunofluorescence, colony formation assay and cell-cycle and apoptosis analysis

Cell lines and culture, dual luciferase assay, quantitative (q)PCR, western blotting and immunofluorescence, colony formation assay and cell-cycle and apoptosis analysis are described in Supporting Information (Material and Methods).

Tissue samples

Thirty-five fresh frozen and 113 formalin-fixed paraffin-embedded GIST tissue samples were obtained from the archives of the Department of Pathology, Yonsei University (Seoul, Korea) and from the Liver Cancer Specimen Bank of the National Research Resource Bank Program of the Korea Science and Engineering Foundation of the Ministry of Science and Technology. The Institutional Review Board of the Yonsei University of College of Medicine approved the use of these tissues for research purposes. All specimens were obtained from patients who had undergone surgical resection at Severance Hospital between 2003 and 2013. None of the patient tissue samples were used in other studies, and no results obtained from these samples have been published elsewhere. Fresh frozen GIST tissues were used for the expression analysis of KIT, miR-494 and survivin using qPCR and western blotting. One hundred and thirteen formalin-fixed paraffin-embedded GIST tissues were used for immunohistochemistry.

Cell proliferation assay

The GIST430 and GIST882 cells were transfected with siNC, miR-494 or siSurvivin. The cells were harvested and manually counted at 3- and 5-day post-transfection. Proliferation and signaling pathway alteration were also evaluated in GIST430 and GIST882 cells with or without kinase inhibitors. The following kinase inhibitors were used in the study: PD0325901 (Sigma, St. Louis, MO, USA), an MEK inhibitor;

ZSTK474 (Cell Signaling Technology, Beverly, MA, USA), a PI3K inhibitor and MK-2206 2HCl (Santa Cruz Biotechnology, Santa Cruz, CA, USA), an AKT inhibitor.

Construction of expression vectors, miRNA mimics, siRNA and transfection

Survivin expression vectors were generated by cloning the coding region (with and without the 3′-UTR region) into a plasmid cytomegalovirus (pCMV, Sigma) vector with a 3×-FLAG tag. The mutant KIT expression vector was described elsewhere.[28] To construct vectors used for target validation and the promoter assays, Target Scan-predicted 3′-UTR regions of survivin, FOXM1, DNMT3B and CKS1B (NCBI reference sequences: NM_001168, NM_202002, NM_001207055 and NM_001826) were conjugated with the Renilla luciferase expression vector (pRL vector, Promega, Madison, WI, USA). The KIT promoter regions (including putative survivin binding sites) were conjugated with the Firefly luciferase expression vector (pGL vector, Promega). MiRNA mimics (miR-221, miR-193a and miR-494), a miR-494 inhibitor and siRNA (siSurvivin) were purchased from Dharmacon (Dharmacon, Lafayette, CO USA). Nontargeting siRNA (siNC), siRNA (siKIT) were purchased from Bioneer (Bioneer Corporation, Daejeon, South Korea). All transfection experiments were performed using lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). Primers used in our study are listed in Supporting Information Table S1.

Chromatin immunoprecipitation (ChiP)

To determine whether survivin binds to the KIT promoter region, ChiP assays were performed using a Simple ChiP Enzymatic Chromatin IP kit (Cell Signaling Technology) according to the manufacturer's instructions. DNA and proteins in GIST430 cells were cross-linked by treatment with 1% formaldehyde. Chromatin was digested using micrococcal nuclease and sonication, and optimal fragmentation was confirmed. Immunoprecipitation was then performed using anti-survivin antibody (Novus Biologicals, Littleton, CO, USA), anti-histone H3 antibody (positive control) and normal rabbit IgG (negative control). The anti-histone H3 antibody and normal rabbit IgG were provided in the ChiP assay kit. Enriched DNA was analyzed using semi-qPCR. RPL30 exon 3 control primers (for the positive control sample) were also provided in the ChiP assay kit. The primers used to amplify the enriched KIT promoter region are listed in Supporting Information Table S1.

Immunohistochemistry and statistical analysis

Tissue microarray sections (4-μm thickness) were obtained from paraffin-embedded GIST tissues from 113 patients. Serial sections were manually stained with antibody against survivin (1:1,200, Cell Signaling Technology). Survivin expression was evaluated based on H-scores, which were calculated using both expression intensity and proportion of expression in tumors according to the following equation: $(1 \times [\% \text{ cells } 1+] + 2 \times [\% \text{ cells } 2+] + 3 \times [\% \text{ cells } 3+])$,

where 1, 2 and 3 represent the relative intensity of survivin expression. H-scores were used to group patients according to strong, moderate or weak survivin expression. Fisher's exact test, the χ^2-test, Kaplan–Meier survival analysis, Pearson's correlation analysis and Cox proportional hazards analysis were used to analyze the relationships between survivin expression and clinicopathological parameters (SPSS software, SPSS, Inc., Chicago, IL, USA). DAVID software (Database for Annotation, Visualization an Integrated Discovery, v6.7; http://david.abcc.ncifcrf.gov) and BINGO 2.3 plugin for Cytoscape software (http://www.psb.ugent.be/cbd/papers/BiNGO/Home.html) were used for pathway and gene ontology analysis. Putative survivin binding regions of KIT gene were selected using MethPrimer (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi).

Microarray and data accession

Total RNA was extracted using an RNA Isolation kit (GE Healthcare Life Sciences, Logan, UT, USA) and then pooled for DNA microarray hybridization. Biotin-labeled cRNA targets were synthesized using 1.5 μg of total RNA, and an Illumina® TotalPrep RNA Amplification kit (Illumina, San Diego, CA, USA) was used to synthesize double-stranded cDNA. Biotin-UTP-labeled antisense RNA was transcribed in vitro using an Ambion MEGAscript kit (Ambion Life Technologies, Carlsbad, CA, USA). All labeling procedures were performed according to the manufacturers' protocols. Microarray experiments were conducted using HumanHT-12 v4 Sentrix Expression BeadChips (Illumina). Hybridization of labeled cRNA to the BeadChip, washing and scanning were performed according to the Illumina Bead Station 500× instructions. Raw data were extracted using the software provided by the manufacturer (Illumina GenomeStudio v2011.1 [Gene Expression Module v1.9.0]). The local-pooled-error (LPE) test was used to determine the statistical significance of differences in expression data, and the false-discovery rate was controlled for given p-values using the Benjamini–Hochberg algorithm. Microarray data are deposited in Gene Expression Omnibus (GEO) with the accession number: GSE89673.

The private accession link:

(https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=qbypgquqtfsxnyb&acc=GSE89673)

Results

MiR-494 downregulates genes involved in the cell cycle and cell cycle-related pathways

We compared the downregulation efficiency of the three previously reported KIT mRNA targeting microRNAs (miR-221, miR-193a and miR-494) in GIST cell lines. Western blot analyses were performed using GIST430 and GIST882 cells treated with the three microRNAs. MiR-494 was the only microRNA that induced significant downregulation of KIT in both GIST430 and GIST882 cells (Fig. 1a). This result was consistent with the results of a previous study.[20] To identify
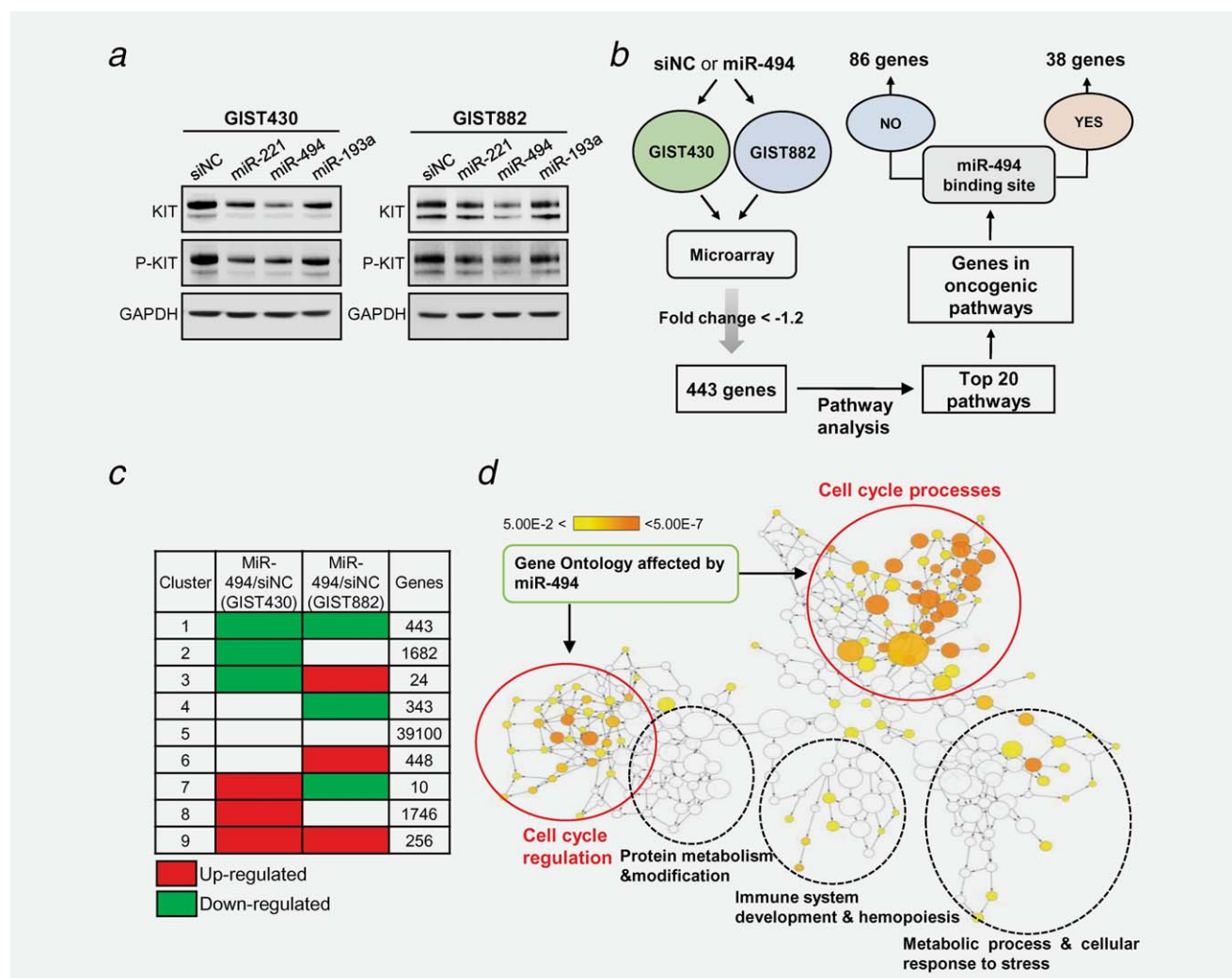
Figure 1. Cell cycle relevant pathways and genes were commonly downregulated by miR-494. (*a*) Downregulation of KIT expression by miR-221, miR-193a and miR-494 was measured using western blot analysis in GIST430 and GIST882 cells. (*b*) A scheme for identifying pathways and genes significantly affected by miR-494 treatment in GIST430 and GIST882 cells. The selected genes were subjected to gene ontology analysis using DAVID software. (*c*) The nine gene expresson patterns found in GIST430 and GIST882 cells after miR-494 treatment. (*d*) Gene ontology network clusters were generated (Cytoscape software) using the genes commonly downregulated by miR-494 in GIST430 and GIST882 cells (443 genes, >1.2-fold downregulation). The significance of enrichment of the corresponding gene ontology is represented by node intensity ($p = 5.00E\text{-}2$, yellow; $p = 5.00E\text{-}7$, orange); the number of genes is represented by node size.

the additional genes affected by miR-494, we performed mRNA expression analysis using two groups each of GIST430 and GIST882 cells, with or without miR-494 treatment. Each group of cells was transfected with siNC or miR-494 and was harvested after 72 hr. The samples of extracted RNA were then subjected to DNA microarray analysis.

The DNA microarray data were used for gene ontology and pathway analysis. A total of 443 genes that were downregulated >1.2-fold in both GIST430 and GIST882 cells were initially selected (Fig. 1*b*). Pathway analysis of the selected genes (using DAVID software) revealed that the cell cycle and cell cycle-related pathways were the most significantly affected by miR-494 treatment (Supporting Information Table S2). This result indicated that inhibition of GISTs by miR-494 treatment was mainly mediated through perturbation of the cell cycle. The

genes in the two cell lines that were affected by miR-494 were categorized into nine expression patterns (Fig. 1*c*); no significant pathway alterations were identified in clusters 2–9 (data not shown). Genes (cluster 1) involved in the cell cycle and cell cycle-related pathways were further selected ($n = 124$). Thirty-eight of the 124 genes had miR-494 binding sites expected by TargetScan (Fig. 1*b*). We further performed gene ontology analysis using an initial selection of 443 genes (>1.2-fold downregulation) with cytoscape and found that the cell cycle relevant categories were mostly changed by miR-494 ($p = 5.00E\text{-}2$, yellow; $p = 5.00E\text{-}7$, orange). Other milder changes (e.g., in protein metabolism & modification, immune system development & hemopoiesis and metabolic process & cellular response to stress) were also identified (Fig. 1*d*). We then validated the microarray results by performing qPCR

analysis of six randomly selected genes. Downregulation of the mRNA expression level of each gene was detected with approximately similar results in both the qPCR analysis and the microarray analysis. This result indicated that the results of the microarray data were valid (Supporting Information Fig. S1).

Survivin, a cell cycle regulator, is a novel target gene of miR-494 in GISTs

We also aimed to identify novel non-KIT genes regulated by miR-494 in GISTs. Based on their reported cancer relevance, we selected CKS1B, DNMT3B, FOXM1 and survivin from the list of the cell cycle-related genes with miR-494 binding sites ($n = 38$); (Fig. 2a). FOXM1 is a transcriptional activator involved in cell proliferation. FOXM1 regulates cell cycle-related genes.[29] DNMT3B is a de novo methyltransferase required for the survival of cancer cells.[30] CKS1B is a regulator that binds to the cyclin-dependent kinases, which promotes G2 to M phase transition.[31] Survivin is also a cell cycle regulator involved in mitosis and has potential clinical significance in GIST tumorigenesis.[32,33] To validate whether these genes were directly regulated by miR-494, reporter vectors were constructed by conjugating each TargetScan-predicted miR-494 binding site located in the 3′-UTR regions of each gene with a luciferase expression vector (Fig. 2b). Reporter assays were performed using HeLa cells transfected with each reporter vector and miR-494. MiR-494 inhibitor treatment was included in each assay to confirm that inhibition of endogenous miR-494 rescues luciferase activity. The reporter assays revealed that luciferase activity from the FOXM1 or DNMT3B reporters was barely changed by the miR-494 or miR-494 inhibitor, while Luciferase activity from CKS1B and survivin reporters was slightly and significantly decreased by miR-494 and increased by the miR-494 inhibitor, respectively (Fig. 2c).

To further validate the reporter assay results, we generated additional mutant CKS1B and survivin reporter vectors by substituting miR-494 site seed sequences with random nucleotide sequences (GTTT to CCGG). In subsequent reporter assays, the decrease in luciferase activity following miR-494 treatment was fully rescued in samples with the survivin mutant vector, but only a slight increase in luciferase activity was observed in samples with the CKS1B mutant vector (Fig. 2d). We therefore found that survivin is a direct and critical target of miR-494 in GISTs. To further demonstrate this finding, we constructed survivin expression vectors with and without the 3′-UTR to confirm miR-494-mediated downregulation of survivin expression (Supporting Information Fig. S2a). Western blotting analysis showed that miR-494 treatment caused a substantial decrease in survivin expression in cells transfected with the 3′-UTR containing the survivin expression vector; however, no change in survivin expression was observed in cells transfected with the survivin expression vector lacking the 3′-UTR (Supporting Information Figs. S2b and S2c).

Next, CKS1B, survivin and miR-494 expression in GIST430 and GIST882 cells was assessed. As GISTs express miR-494 at very low levels, high levels of survivin and CKS1B expression were expected.[16] Western blot analyses revealed only low-level expression of CKS1B, whereas survivin was expressed at high levels (Fig. 2e). Further analysis of miR-494 expression confirmed the inverse relationship between miR-494 and survivin expression in GIST430 and GIST882 cells (Fig. 2f).

Based on a report indicating that survivin expression decreases upon KIT inhibition, we investigated whether miR-494 represses survivin expression independent of KIT.[33] As previously reported, RNAi-mediated KIT depletion induces a significant decrease in survivin expression, which is almost completely rescued by KIT overexpression. Notably, we found that the decrease in survivin expression following KIT depletion by miR-494 treatment was not rescued, which suggests that miR-494 independently downregulates survivin expression irrespective of changes in KIT expression (Supporting Information Fig. S2d).

Survivin expression is inversely correlated with miR-494 expression in GIST tissues

After we found that survivin mRNA is a direct target of miR-494, we examined the relationships in expression between survivin and miR-494 in human GIST tissues. We measured miR-494 expression using Taqman-based microRNA qPCR and survivin expression, and western blot analysis, in 35 GIST tissues. KIT expression was also measured to confirm the previously reported inverse correlation between miR-494 and KIT in GISTs (Fig. 3a).[20] Levels of KIT and survivin expression were normalized to GAPDH expression, and miR-494 expression was normalized to that of RNU6B. The resulting normalized expression data were log–log converted for subsequent statistical analyses (Supporting Information Fig. S3). The correlation analysis revealed that KIT expression was inversely correlated with miR-494 expression in the 35 GIST tissues (Pearson's correlation coefficient, $r = -0.450$, $p = 0.007$) (Fig. 3b). MiR-494 expression was inversely correlated with survivin expression (Pearson's correlation coefficient, $r = -0.418$, $p = 0.012$) (Fig. 3c). A tendency toward a positive correlation was also found for KIT and survivin expression, but this result was not statistically significant (Pearson's correlation coefficient, $r = 0.267$, $p = 0.120$) (Fig. 3d). This lack of statistical significance may be due to the additional factors (e.g., ETV1 and PKC-θ) that can affect KIT expression.[28,34]

Inhibition of survivin leads to G2/M phase arrest and subsequent apoptosis, accompanying perturbation of the PI3K-AKT pathway

To investigate the biological significance of survivin in GISTs, we performed a proliferation assay using GIST430 and GIST882 cells transfected with siNC, miR-494 or siSurvivin. siSurvivin was included to determine whether KIT-independent
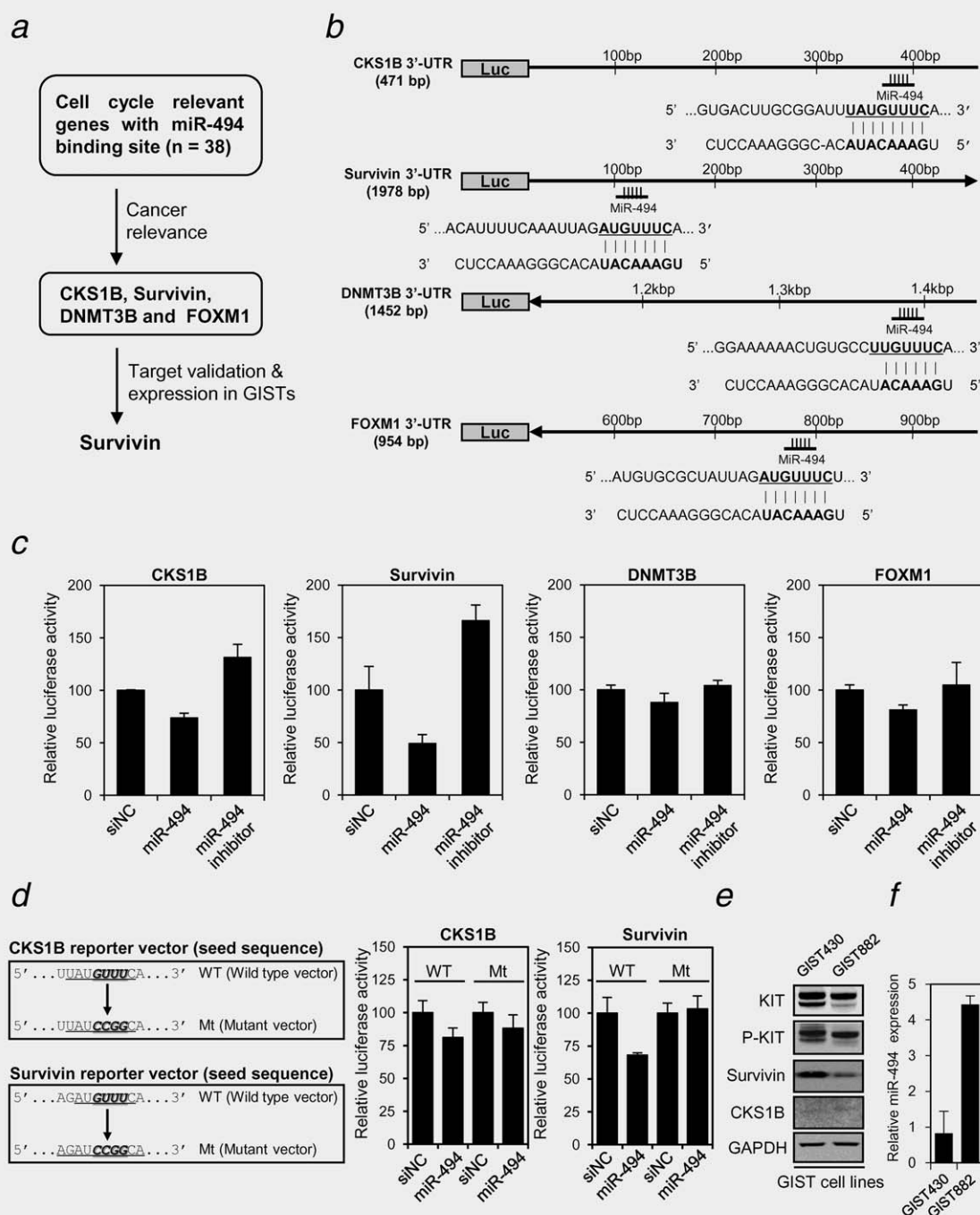
Figure 2. MiR-494 directly regulates survivin, which is universally overexpressed in GIST cells. (*a*) Putative novel miR-494 target genes were selected (*CKS1B*, *survivin*, *DNMT3B* and *FOXM1*) based on previous reports and cancer relevance. Survivin was selected after target validation assay, and endogenous protein expression level, results. (*b*, *c*) The reporter vector was constructed by conjugating Renilla luciferase with a Target-Scan predicted miR-494 binding site in each 3′-UTR of *CKS1B*, *survivin*, *DNMT3B* and *FOXM1*. Each reporter vector with siNC, miR-494 or miR-494 inhibitor was transfected into HeLa cells. Dual luciferase assays were performed by measuring Renilla and Firefly luciferase activity at 48 hr post-transfection. (*d*) Mutant reporter vectors for CKS1B and survivin were further constructed by substituting seed match sequences of miR-494 (GTTT->CCGG). After transfection of the wild-type reporter vector (WT) and mutant reporter vector (Mt) with siNC or miR-494, a dual luciferase assay was performed. WT denotes the wild-type vector with the orginal binding site of miR-494. Mt denotes the mutant vector with a mutated binding site of mR-494. To determine the relative luciferase activity, the Renilla luciferase activity was normalized based on the Firefly luciferase activity. (*e*, *f*) Endogenous survivin, CKS1B and miR-494 expression analyzed by western blotting and qPCR analysis, respectively, in GIST430 and GIST882 cells.
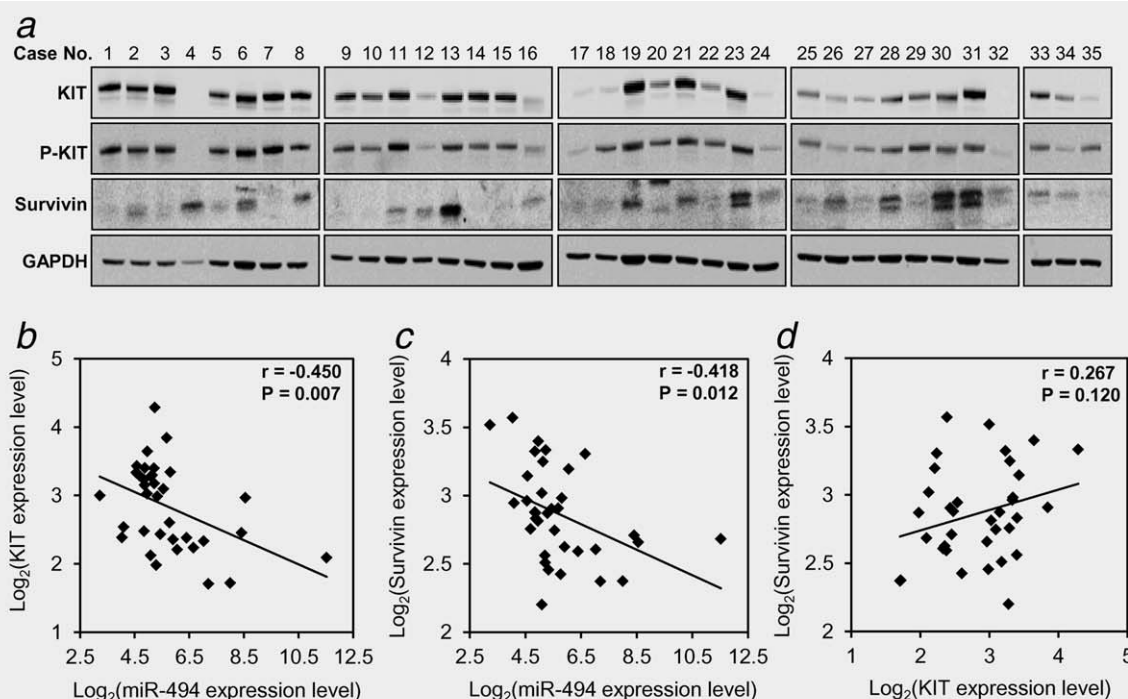
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Figure 3. Relationships between the expression of survivin, miR-494 and KIT in 35 GIST tissues. (*a*) The expression levels of KIT, P-KIT, survivin and GAPDH were mesured in 35 GIST tissues using western blot analysis. (*b–d*) Statistical correlation analysis was performed for the expression of KIT and miR-494 (Pearson's correlation coefficient, $r = -0.450$, $p = 0.007$), the expression of survivin and miR-494 (Pearson's correlation coefficient, $r = -0.418$, $p = 0.012$) and the expression of KIT and survivin (Pearson's correlation coefficient, $r = 0.267$, $p = 0.120$).

survivin downregulation resulted in the inhibition of GIST cells. The GIST cells were counted and harvested at 3- and 5-day post-transfection. Western blot analysis was performed to evaluate the knock-down efficiency of microRNA and RNAi. We found that miR-494 treatment efficiently inhibited proliferation of the GIST430 and GIST882 cells, with concomitant downregulation of KIT and survivin. The similar pattern of proliferation inhibition was observed when survivin was depleted by siRNA (Fig. 4*a*). FACS analysis of the cell cycle after miR-494 or siSurvivin treatment revealed that miR-494 induced a slight increase (5.9% in GIST430 and 4.7% in GIST882 cells) in the G0–G1 phase and a decrease (2.5% in GIST430 and 0.6% in GIST882 cells) in the S phase. Survivin knock-down induced substantial increases (26.1% in GIST430 and 27.9% in GIST882 cells) in the G2-M phase and substantial decreases (27.9% in GIST430 and 30.9% in GIST882 cells) in the G0–G1 phase (Fig. 4*b*). Subsequent apoptosis analysis revealed that early and late apoptosis were significantly induced in both GIST cell lines by miR-494 (19.9% in GIST430 and 16.6% in GIST882 cells) and by siSurvivin (22.5% in GIST430 and 30.8% in GIST882 cells) (Fig. 4*c*). MiR-494- and siSurvivin-mediated GIST inhibition was further revealed by the colony formation assay. Two weeks after transfection of miR-494 or siSurvivin into each cell line, colonies with >50 cells were counted. Colony numbers were markedly reduced by miR-494 (73.2% in GIST430 and 51.3% in GIST882 cells) or siSurvivin (97.3% in GIST430 and 96.9% in

GIST882 cells) treatment (Fig. 4*d*). Overall, KIT-independent downregulation of survivin was sufficient to inhibit GISTs, with accompanying dramatic cell cycle arrest and cell death.

In addition to survivin's roles during the cell cycle process, it is also an effector molecule belonging to the mTOR pathway, which is a subpathway of the PI3K-AKT pathway.[35,36] Therefore, we examined whether inhibition of survivin would affect the PI3K-AKT pathway in GISTs. The levels of the activated forms of AKT, ERK and STAT3 were measured after miR-494 or siSurvivin treatment in GIST430 and GIST882 cells. MiR-494 and siSurvivin treatment induced slight decreases in phospho-STAT3 and substantial decreases in phospho-AKT. The phospho-ERK levels were not changed by exposure to miR-494 or siSurvivin (Fig. 5*a*). To further examine the significance of the PI3K pathway in GISTs, GIST cells were exposed to specific kinase inhibitors against MEK (PD0325901), PI3K (ZSTK474) or AKT (MK-2206) and proliferation assays were performed. The proliferation assay revealed that the inhibitor against the PI3K pathway significantly blocked GIST proliferation; inhibitors against the MEK or AKT pathways were less, or were not, effective (Fig. 5*b*). Alteration of downstream signaling molecules after treatment with PI3K, AKT or MEK inhibitors was also analyzed using western blot. Prolonged treatment with inhibitors against AKT and PI3K induced dramatic downregulation of survivin; the inhibitor against MEK barely affected survivin
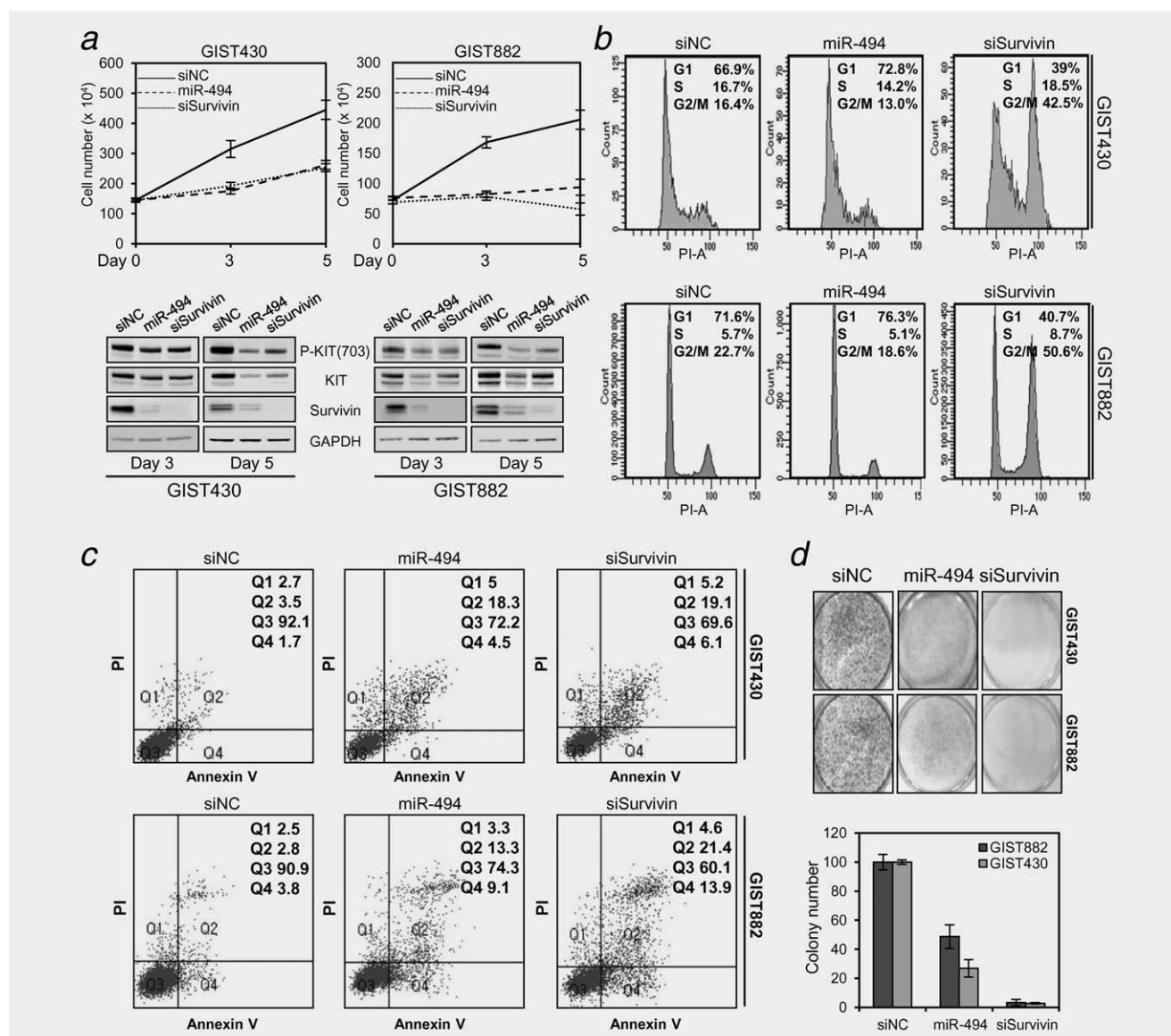
Figure 4. Downregulation of survivin inhibits proliferation of GIST cells via the perturbation of the cell cycle. (*a*) Proliferation of GIST430 and GIST882 cells after exposure to miR-494 or siSurvivin was measured by manually counting cells at 3- and 5-day post-exposure. Knockdown efficiency of miR-494 and survivin was confirmed using western blot analysis of KIT and survivin. (*b*, *c*) Cell-cycle (propidium iodide staining) and apoptosis (Anexin and propidium iodide staining) analysis in GIST430 and GIST882 cells were performed after exposure to miR-494 or siSurvivin. (*d*) Colony formation of GIST430 and GIST882 cells was evaluated after miR-494 or siSurvivin treatment. Two weeks after seeding 40,000 cells of GIST430 and GIST882, colonies were stained using crystal violet and were counted using a Colony V1.1.

(Fig. 5*c*). These findings verified the significance of the PI3K pathway in GISTs. The results also indicated that survivin was regulated by the PI3K-AKT pathway and inversely regulated the PI3K-AKT pathway.

Overexpression of survivin relieves miR-494-mediated GIST inhibition

We next evaluated whether survivin overexpression could overcome the miR-494-mediated inhibition of GIST cells. A proliferation assay was performed using GIST cells transfected with miR-494 only or with miR-494 with the survivin expression vector. The cells were counted at days 3 and 5

after transfection. We found that the floating dead cells and cells with abnormal morphology caused by miR-494 were rarely observed when survivin was overexpressed in the GIST cells (Supporting Information Fig. S4). The proliferation assay revealed that miR-494-mediated GIST inhibition was substantially rescued by survivin overexpression in the GIST430 and GIST882 cells (Fig. 5*d*). Constant downregulation of survivin by miR-494 and the restored expression of survivin by the survivin expression vector were confirmed by western blot analysis (Fig. 5*e*). Therefore, combined inhibition of survivin and KIT would be a more effective approach for a GIST subtype expressing KIT and survivin.
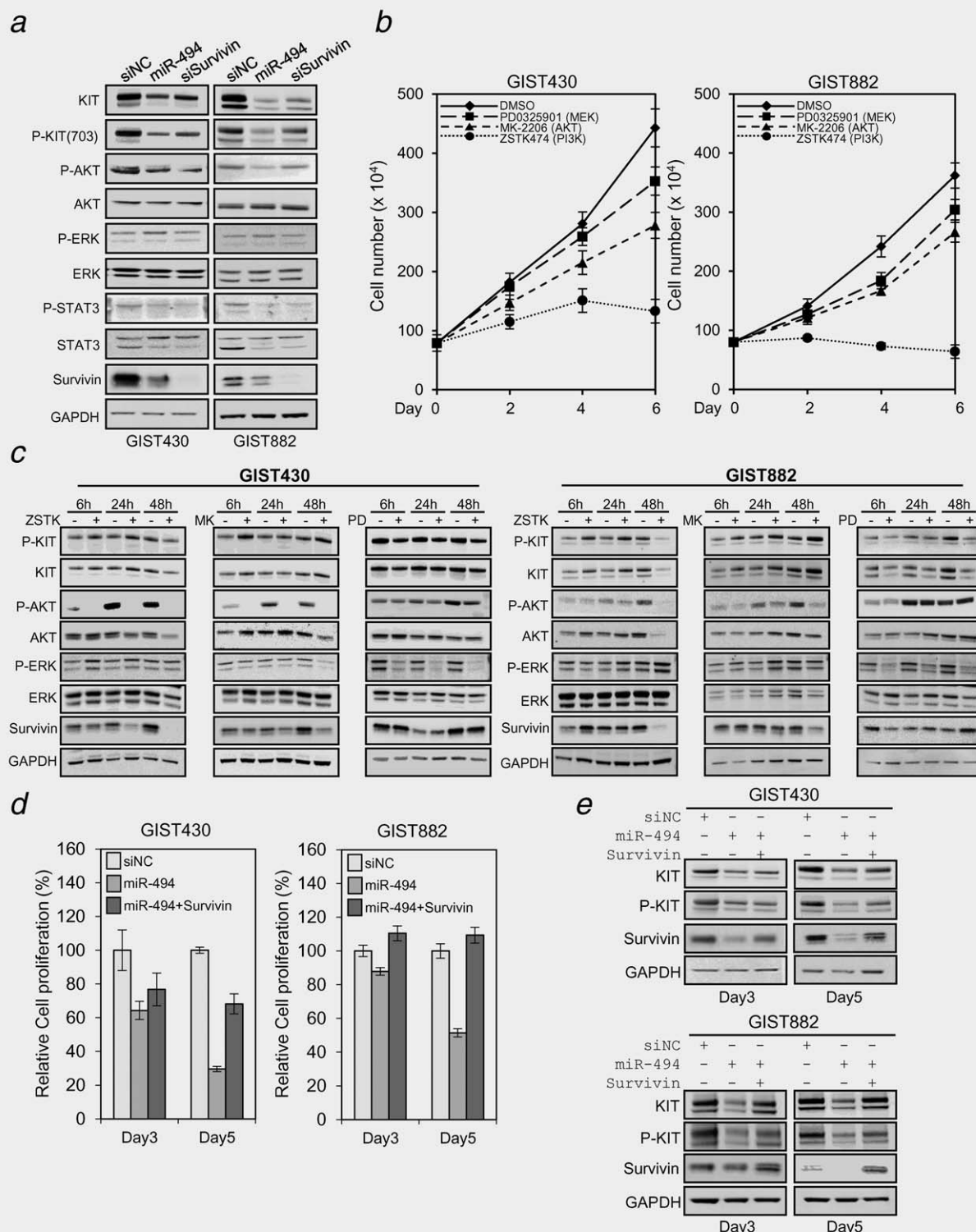
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Figure 5. Downregulation of survivin induced the inhibition of the PI3K-AKT pathway and overexpression of survivin rescued miR-494-mediated GIST inhibition. (*a*) The expression of KIT, p-KIT, AKT, p-AKT, p-STAT3, STAT3, p-ERK, ERK and survivin in GIST430 and GIST882 was measured after treatment with siNC, miR-494 or siSurvivin. (*b*) The inhibitory effect on GIST430 and GIST882 cells was evaluated by comparing proliferation of GIST cells with or without treatment with specific inhibitors against MEK (PD0325901, 100 nM), PI3K (ZSTK474, 10 μm) or AKT (MK-2206, 5 μm). Cells were counted at 2 days, 4 days and 6 days after inhibitor treatment. (*c*) The changes in expression of p-KIT, KIT, p-AKT, AKT, p-ERK and ERK, and survivin, were measured using western blot analysis performed after time-course treatment with each inhibitor. (*d*) The cell numbers were normalized to the control cell numbers and presented as relative values. (*e*) The rescued expression of survivin by transfection of the survivin expression vector with miR-494 was confirmed using western blot analysis.

Survivin is mainly localized in the nucleus and regulates KIT mRNA expression by binding the promoter region of *KIT*

When survivin expression was manipulated by transfecting the survivin expression vector or siSurvivin, we found that KIT protein expression changed in response (Figs. 4a, 5a and 5e). We therefore hypothesized that survivin might regulate *KIT* expression, possibly at the mRNA or protein level, or both. To investigate how survivin regulated *KIT* expression, the localization of KIT and survivin was analyzed using immunofluorescence microscopic and immunohistochemistry examination in GIST cells and tissues. As previously reported, KIT showed perinuclear localization, which represented its retention in the Golgi complex.[28] However, survivin was detected in the nucleus. These results indicated that there was limited direct interaction between KIT and survivin. Because survivin was only detected in the nucleus, we suspected that survivin might regulate *KIT* expression at the transcription level (Fig. 6a). Thus, we measured the mRNA and protein expression of *KIT* in GIST cells after transfection of the survivin expression vector or siSurvivin. Western blot analysis revealed that induced overexpression of survivin resulted in a gradual increase in KIT expression in both GIST430 and GIST882 cells (Fig. 6b). The results of the qPCR analysis indicated that the KIT mRNA expression similarly increased along with the gradual increase in KIT expression (Fig. 6c). Moreover, siRNA-mediated downregulation of survivin induced a significant decrease in KIT mRNA expression, and a subsequent reduction in KIT protein expression, in both GIST cell lines (Figs. 6d and 6e).

We also investigated changes in KIT mRNA and protein expression after survivin downregulation/overexpression in two colon cancer cell lines (DLD-1 and Colo320DM) that express wild-type KIT. Western blot and qPCR analyses showed that downregulation of survivin induced decreases in KIT mRNA and protein levels in both cell lines (Supporting Information Figs. S5a and S5b), whereas overexpression of survivin resulted in increased KIT mRNA and protein expression (Supporting Information Figs. S5c and S5d). These findings allowed us to suspect the putative roles for survivin as a transcription regulator for *KIT*. To test survivin protein interact with which promoter region of *KIT*, reporter vectors containing the promoter regions of *KIT* were generated. We divided the promoter region ($\sim$2 Kb) of *KIT* into four fragments by calculating the expected score of transcription factor binding obtained from a bioinformatic software program (MethPrimer). Each reporter vector was transfected into HeLa cells with the survivin expression vector and was harvested to measure luciferase activity. We found that compared to the control, the vector containing fragment 3 (-329 to $+155$, the flanking region of exon 1) had a dramatic increase in luciferase activity in response to the survivin overexpression ($>$20-fold); no significant increases in luciferase activity occurred in the vectors containing the other fragments (Fig. 6f).

A ChiP assay was conducted to further confirm the direct interaction between survivin and the *KIT* promoter fragment 3 regions. Chromatin samples were incubated with anti-survivin antibody, anti-histone H3 antibody (positive control) and IgG (negative control). Semi-qPCR analysis using the isolated chromatin samples showed that the flanking region of exon 1 (-47 to $+123$) was most significantly enriched by ChiP. These findings were consistent with the reporter assay results (Figs. 6g and 6h) and indicate that survivin directly binds to the *KIT* promoter region, enhancing the transcriptional activity of *KIT*.

The expression of MiR-494 and survivin is related to the poor clinical outcome of GIST patients

We evaluated the clinical behavior of GISTs according to survivin expression by performing immunohistochemistry for survivin using 113 GISTs. Survivin expression was categorized as a strong ($n = 8$), moderate ($n = 49$) or weak ($n = 56$) response (Supporting Information Fig. S6a). Kaplan–Meier survival analysis revealed that the patients with strong expression of survivin had poor overall survival ($p = 0.004$) (Supporting Information Fig. S6b). We further analyzed the relationships between survivin expression and the clinical parameters, depending on the mutation status of *KIT* in 113 GISTs. Survivin expression was closely related to mitotic index ($p = 0.013$), tumor size ($p = 0.018$) and poor patient survival ($p = 0.016$) in a cohort of GIST patients with the KIT mutation ($n = 93$) (Supporting Information Table S3). Multivariate analysis indicated that strong expression of survivin ($p = 0.025$) was a predictive factor for the poor patient survival (Supporting Information Table S4).

The relationship between miR-494 expression and clinical characteristics of tumors was analyzed relative to miR-494 expression in the 35 GISTs described in Figure 3. Due to the lack of follow-up data for 1 patient, data for only 34 of the GISTs were statistically analyzed. The 34 cases were classified as strong ($n = 8$), moderate ($n = 18$) or weak ($n = 8$) miR-494 expression. Statistical analyses showed correlations between miR-494 expression and tumor size ($p = 0.001$), mitotic index ($p = 0.024$), grade ($p = 0.003$) and survival ($p = 0.013$) (Supporting Information Table S5). Kaplan–Meier analysis of the data revealed that patients expressing weak levels of miR-494 had poorer overall survival ($p = 0.017$) (Supporting Information Fig. S7). However, multivariate analysis of the 34 GIST cases revealed no significant predictive factors for survival or recurrence/metastasis (Supporting Information Table S6).

Discussion

Progression on imatinib in metastatic patients with GIST is associated with a mostly dismal outcome and salvage therapies have failed to overcome resistance.[10,11] ATP-competitive inhibitors of KIT are unlikely to inhibit the full spectrum of resistance mutations and therefore alternative measure might be useful to inhibit oncogenic KIT signaling. A number of
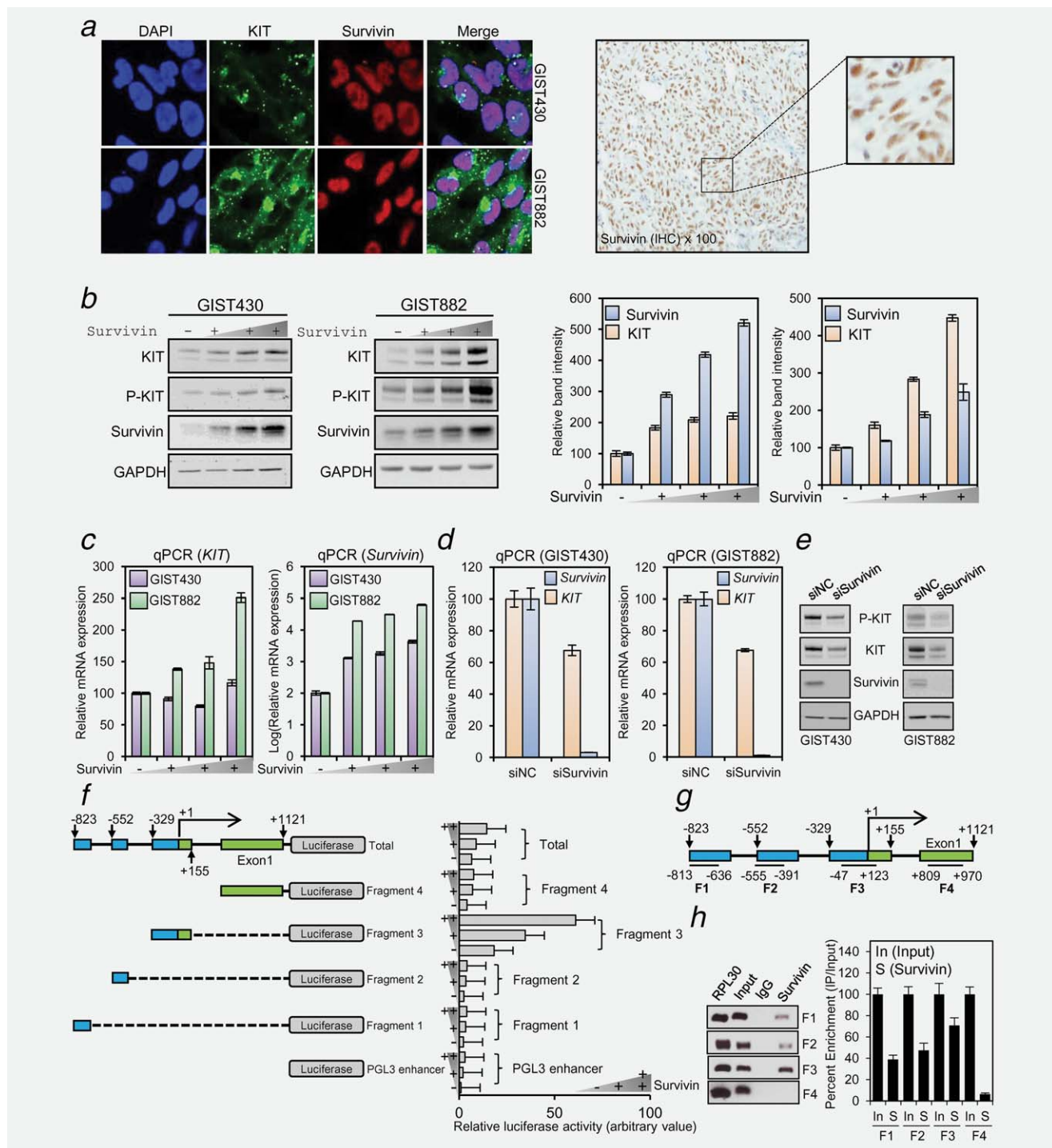
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Figure 6. Survivin binds to a specific region of the *KIT* promoter and induces *KIT* expression at the transcription level. (*a*) The localization of KIT and survivin was examined using GIST430 and GIST882 cells and immunofluorescence microscopic analysis (left panel). The nuclear localization of survivin was examined by immunohistochemistry in a representative GIST tissue (right panel). (*b*, *c*) The changes in expression of *KIT* mRNA and protein were evaluated using qPCR and western blot in GIST430 and GIST882 cells transfected with gradually increasing amount of survivin plasmids. (*d*, *e*) Expression of *KIT* mRNA and protein were measured after siSurvivin treatment. (*f*) To map a specific region of *KIT* promoter bound by survivin, various *KIT* promoter fragments conjugated with Firefly luciferase (five different fragments, including the full-length promoter) were constructed, and dual luciferase assay was performed 48 hr after tranfection of each construct with survivin expression vector into HeLa cells. The schematic map of the vectors is presented on the left panel and the relative luciferase activity results are presented on the right panel. The *KIT* promoter region was obtained from bioinformatic software (MethPrimer). To determine the relative luciferase activity, the Firefly luciferase activity was normalized based on the Renilla luciferase activity. (*g*) Chromatin immunoprecipitation assay performed using anti-survivin, anti-histone H3 and normal rabbit igG antibodies. (*h*) Enrichment of the survivin-binding DNA fragment in the *KIT* promoter region was evaluated by semi-qPCR. In the positive control sample, binding of histone H3 to *RPL30* exon 3 was evaluated (left panel). Densitometric analysis of the intensity of semi-qPCR assay bands (right panel).

investigators have tried to improve delivery of conventional drugs, and develop a new generation of drugs that include small RNA-based therapeutic tools. Deacetylase inhibitors have previously been shown to downregulate KIT both by transcriptional repression and by HSP90-mediated degradation with evidence of clinical activity.[37,38] One of the breakthroughs in GIST inhibition has been the identification of microRNAs showing inverse correlation with KIT expression in GISTs.[17,19–21]

The gene ontology analyses using microarray data revealed that the pathways affected by miR-494 were mostly relevant to the cell cycle. This result suggested that miR-494 specifically targeted a group of cell cycle relevant genes. Concomitant regulation of cell cycle relevant genes by miR-494 led to the arrest of the overall cell cycle, and eventual cell death. These findings were consistent with the results of studies that some genes targeted by a specific microRNA tend to be functionally linked; altered expression of a single microRNA might cause severe effects on a specific cellular pathway.[22] Among the cell cycle relevant genes identified in our study, survivin is of particular interest because it is involved in the AKT-mTOR pathway, which is critical for GIST tumorigenesis and therapy.[6,36] Upregulation of survivin disturbs the anti-cancer effects of several inhibitors against upstream molecules involved in the IGFR/PI3K/AKT/mTOR pathway in hepatocellular carcinoma.[35] However, a complete understanding of the details of the roles of survivin in GISTs has not been elucidated. In our study, we found that survivin mRNA was a direct target of miR-494 and that survivin expression was inversely correlated with miR-494 expression. Evaluation of cell proliferation, the cell cycle, apoptosis and colony formation, also revealed that downregulation of survivin was sufficient to inhibit GISTs without manipulation of KIT. We also found that survivin overexpression rescued miR-494-mediated GIST inhibition. These findings suggest that survivin has critical roles in GISTs and is as important a target of miR-494 as KIT is, for GIST inhibition.

Survivin has various roles depending on its location in the cell. Nuclear survivin binds to the mitosis promoting complex and induces cell cycle progression.[32] Mitochondrial survivin reportedly binds directly to caspases 3 and 7, inhibiting the caspase-mediated cascade leading to apoptosis.[39,40] The observation that survivin also functions as a downstream effector in the PI3K-AKT pathway also suggests it inhibits apoptosis.[35] However, some studies have reported controversial findings suggesting that the apoptosis-inhibiting role of survivin varies with different types of cancer.[41,42] The survivin gene was recently identified as essential for survival of GISTs in a synthetic lethality screen; YM155-mediated survivin inhibition leads to strong apoptosis induction.[33] Considering these reports in conjunction with our finding that survivin depletion significantly inhibits GIST cell proliferation and promotes apoptosis in GIST cells, we hypothesize that survivin, particularly its anti-apoptotic effect, plays an important role in GIST tumorigenesis and therefore warrants further characterization.

In our study, survivin was detected only in the nucleus, and manipulation of survivin resulted in significant changes in *KIT* expression. Using overexpression and downregulation of survivin in GIST and colon cancer cell lines, we found that KIT mRNA expression was regulated by survivin. Sp1, GATA and SCL have been identified as transcriptional regulators of *KIT* in mast cells and hemopoietic cells; only ETV1 has been reported to regulate *KIT* expression at the transcription level in GIST cells.[34,43,44] To our knowledge, this is the first study to find that survivin is a transcriptional regulator of *KIT*. Survivin directly interacted with the promoter region near exon 1 of *KIT* and upregulated KIT mRNA expression. These findings indicate that (1) survivin is a novel transcriptional regulator of *KIT*, and (2) the two target genes of miR-494, *KIT* and *survivin*, form an activation loop that contributes to GIST progression.

Sustained activation of KIT leads to the hyperactivation of the MAPK, PI3K and JAK/STAT pathways.[6,45] However, preclinical studies have found that inhibiting the PI3K/AKT/mTOR pathway is a rational method to overcome the resistance associated with prolonged treatment.[6,46,47] Consistent with previous study results, we found that the inhibitor against the PI3K pathway had most potent and long-term inhibitory effect on GIST proliferation. However, many studies have also found that single molecule targeting cancer treatment is challenging due to the compensatory activation of other molecules belonging to the same pathway. For example, targeting a single effector molecule belonging to the EGFR-Ras-Raf-MEK-ERK pathway almost always leads to drug resistance in colon cancers.[48] In addition, compensatory activation between the PI3K pathway and the mTOR pathway has also been reported in prostate and breast cancer cell lines.[49]

Several studies have suggested the use of combined therapeutic approaches based on the idea that targeting multiple effector molecules enhances the therapeutic effects of anti-cancer agents. Zhu *et al.* demonstrated that combined use of miR-494 and RNAi targeting survivin synergistically and markedly inhibit the growth of prostate cancer cells.[50] The vertical blockade combined therapeutic approach targets effector molecules within the same signaling pathway, leading to potent and prolonged inhibition of cancer cell growth. Single microRNAs that target genes belonging to functionally linked groups are potentially ideal vertical blockade agents.[22] Our findings that KIT and survivin are functionally linked targets of regulation by miR-494 or the PI3K pathway in GISTs suggest that miR-494 could be used as a novel vertical blockade agent for treating GISTs (Supporting Information Fig. S8).

Author's Contributions

Conception and design: H.K., S.Y. and W.K. Development of methodology: S.Y. and W.K. Data acquisition: S.Y., W.K.,

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M.J. and Y.K. Data analysis and interpretation: S.Y., W.K. and H.K. Manuscript writing, review and/or revision: S.Y., H.K. and S.B. Administrative, technical or material support: H.K. and S.B. Study supervision: H.K.

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