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RESEARCH ARTICLE

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GDF15 induced apoptosis and cytotoxicity in A549 cells depends on TGFBR2 expression

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GDF15 plays a paradoxical role during carcinogenesis; it inhibits tumour growth in the early stages and promotes tumour cell proliferation in the late stages of cancer. Besides, GDF15 can induce apoptosis in some cancer cells including A549 but not in some others. Moreover, as a potential receptor for GDF15, TGFBR2 is inactivated during carcinogenesis in many types of cancers, and it is not present in cells with no GDF15 induced apoptosis. Thus, we tested whether GDF15 overexpression and/or TGFBR2 silencing can affect the GDF15 induced apoptosis in A549 cells. The full and mature forms of GDF15 were cloned and overexpressed in A549 cells. The TGFBR2 was silenced using specific siRNA and confirmed by real-time PCR. Results indicated that overexpression of full and mature forms of GDF15 as well as TGFBR2 knocked down reduced A549 cell viability in 24 and 48 hours. Flow cytometric analysis of annexin V/PI indicated induction of apoptosis in A549 cells by overexpression of GDF15 or silencing TGFBR2. Interestingly, the silencing of TGFBR2 inhibited the GDF15 induced cytotoxicity and apoptosis in A549 cells. Overexpression of GDF15 activated caspase-9 and caspase-3 and inhibited ERK1/2 and p38 phosphorylation in A549 cells. TGFBR2 knocked down inhibited GDF15 effects on caspases, ERK1/2, and p38MAPK activation. Our results indicated that the effect of GDF15 on apoptosis and activation of MAPK in A549 cells depends on TGFBR2 expression. These findings may point to mechanisms in which GDF15 exerts dual effect during carcinogenesis with regard to TGFBR2 expression.

Significance of the study: GDF15 plays a tumour suppressor or promotor roles during carcinogenesis. The expression of GDF15 induced cytotoxicity, apoptosis, and inhibition of MAPK in A549 cells. All these effects were blocked by silencing TGFBR2 expression. These findings may point to mechanisms in which GDF15 exerts dual effect during carcinogenesis with regard to TGFBR2 expression.

KEYWORDS

apoptosis, GDF15, lung adenocarcinoma, MAPK, NAG1, TGF-β receptor

1 | INTRODUCTION

Growth differentiation factor 15 (GDF15) was identified by independent research groups in the late 1990s,[1] and it has taken different names including NAG-1, MIC-1, PLAB, PDF, PTGFB, and NRG1. It is recently suggested to consider GDF15 as a member of the GDNF family because GDF15 binds specifically to GDNF family receptor a-like (GFRAL) with high affinity and its co-receptor RET.[2] GDF15 gene is

located on chromosome 19p12-13.1 and encodes two propeptides of 308 or 295 amino acid.[1,3] Usually, the GDF15 protein is produced as a 308-amino-acid propeptide (62 kDa), and after intracellular cleavage by a furin like protease at a conserved RXXR motif, the mature protein is secreted as a 112-amino-acid homodimer (25 kDa). The disulfide-linked homodimer contains seven conserved cysteines which are necessary for the cysteine knot formation.[1,4] Multiple forms of GDF15, including pro-GDF15 monomer, pro-GDF15 dimer, pro-peptide N-terminal fragment, and mature dimer are present in cells. It has been reported that the multiple forms of endogenous GDF15 may mediate different functions; however, the mature protein is probably the main bioactive form.[3,4] It remains unclear whether the other forms are biologically active, or whether the different forms affect the biological behaviour of the cells.

GDF15 plays multifunctional roles in context of diverse physiological and pathological conditions including proliferation, apoptosis, senescence, stemness, obesity, inflammatory response, and malignancies. It has been shown that in the early stage of cancer, GDF15 can induce tumour cells apoptosis, and at late stages, GDF15 production will help metastases and tumour progression.[3,5-7] It has been reported that GDF15 contributes to induce apoptosis in various cell lines including A549 lung adenocarcinoma,[8,9] and lower level of GDF15 in malignant gliomas has reduced cell proliferation and tumorigenesis,[10] suggesting the contribution of GDF15 to cancer progression. Interestingly, in the oral squamous cell carcinoma (OSCC), the TGFBR2 is downregulated,[11] and GDF15 in OSCC has no apoptotic effect and inhibits caspase activity.[12] Thus, as a potential receptor for GDF15, it is rational to consider the link between expression level of TGFBR2 and the GDF15 effect on apoptosis. In this regards, Artz et al have suggested TGFBR2 as a receptor for GDF15 effect in immune system, and they have shown that ALK-5/TGFBR2 heterodimer can mediate the effect of GDF15 on integrin activation and neutrophil recruitment in mice.[13] TGFBR2 is a tyrosine kinase receptor located in the cell membrane and forms heteromeric receptor complex with TGFBR1 to initiate the signal transduction of TGFBR1.[14] Many studies have shown that TGFBR2 is inactivated and/or suppressed during carcinogenesis in various types of tumours.[15,16] Thus, it is logical to consider the TGFBR2 level related to the effects of GDF15 in different cells. Although the role of GDF15 on cell behaviour during tumour development has been evaluated, further studies are needed to assess the functional differences and the underlying mechanism of GDF15 effects in cancer cells. Therefore, we have addressed the effect of full and mature forms of GDF15 on proliferation and induction of apoptosis considering the role of TGFBR2 receptor as well as signalling of p38 and ERK pathways in A549 cells.

2 | MATERIALS AND METHODS

2.1 | Cell culture

A549 cells, human lung adenocarcinoma epithelial cell line, were obtained from the Iranian Biological Resource Center (IBRC; http://en.ibrc.ir/, accession cell no: IBRC C10080) and cultured according to standard mammalian cell culture protocols. The cells were cultured in DMEM/F12 medium (Biowest, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, UK), 100 U/mL penicillin and 100 μg/mL streptomycin (PAA, UK) at 37°C in a humidified atmosphere containing 5% CO_2.

2.2 | RNA purification and cDNA preparation

Total RNA was isolated from A549 cells and human peripheral blood mononuclear cells (PBMC) using Tripure Isolation Reagent (Roche, Germany) according to the manufacturer's protocol. The concentration and purity of the RNA samples were determined using spectrophotometer (260/280 nm) and visualization of 18S and 28S RNA bands on agarose gel electrophoresis. For cDNA synthesis, 500 ng of total RNA was reverse transcribed using PrimeScript RT reagent Kit (Takara, Japan) according to the manufacturer's instructions.

2.3 | Designing and cloning of full and mature GDF15 expression constructs

The cDNA for full GDF15 coding region was amplified using Pfu DNA Polymerase (GeneAll, Korea), with specific forward and reverse primers containing HindIII and XbaI restriction site, respectively (Table 1). The mature GDF15 coding sequence including the C-terminal 112 amino acids of full length protein was amplified using specific forward and reverse primers containing KpnI and XbaI cleavage site, respectively (Table 1). A start codon and Kozak sequence were engineered into mature GDF15 forward primer for proper expression of protein. The stop codon was removed from reverse primers to genetically fuse with the His-tag sequence on vectors. The PCR products were digested with appropriate restriction endonucleases (Fermentas & Takara) and ligated into pcDNA3.1 myc-His A. The constructs were transformed into *Escherichia coli* (DH5α), and the plasmids were purified using the Genopure Plasmid Maxi Kit (Roche, Germany) according to the manufacturer's instruction. The constructed plasmids were verified by restriction digests and DNA sequencing for the orientation, frame, and sequences.

2.4 | Design and transfection of synthetic siRNAs

Specific siRNA oligonucleotide targeting TGFβR2 based on the human TGFBR2 mRNAs (NM_003242.5, NM_001024847.2), scrambled nontargeting siRNA, and fluorescent siRNA were designed (Table 1) and synthesized (Bioneer, Korea). To enhance siRNA stability and effectiveness, 5-phosphorylation and 3-dTdT overhang modification was applied. A549 cells were plated 24 hours, and at confluency of 70% to 80%, cells were transfected with 20-nM siRNA using Lipofectamine LTX reagent (Invitrogen, USA), according to the manufacturer's instruction. The cells were harvested 24 hours after transfection for analyses, and untransfected A549 cells were used as controls.

TABLE 1 List of primer and siRNA sequences used in the study

Primer Name	Sequence	Comments
FN1-F	CTCAGAGCCGCAACCTGCACAG	Cloning
FN1-R	TGCACAGTGGAAGGACCAGGACTG	Cloning
FN1-Hi3-F	CCGCAAGCTTCACAGCCATGC	Cloning
FMN1-Xb-R	GAAGGTCTAGAATCTGCTTCTATGCAGTGG	Cloning
MN1-Kp-F	AAGGTACCATGGCGCGTGCG	Cloning
GDF15-F	CGGAAACGCTACGAGGACCTG	Real-time PCR
GDF15-R	GAGAGATACGCAGGTGCAGGTG	Real-time PCR
TGFBR2-F	TCGAAAGCATGAAGGACAACG	Real-time PCR
TGFBR2-R	AGCACTCAGTCAACGTCTCAC	Real-time PCR
GAPDH-F	GAAGGTGAAGGTCGGAGTCAAC	Real-time PCR
GAPDH-R	CAGAGTTAAAAGCAGCCCTGGT	Real-time PCR
HPRT-F	CTGGCGTCGTGATTAGTGATGATGA	Real-time PCR
HPRT-R	TCGAGCAAGACGTTCAGTCCTGTC	Real-time PCR
siRNA Name	Target Sequences (5′ to 3′)	siRNA Duplex Sequences (5′ to 3′)
TGFBR2 siRNA	AAGGACATCTTCTCAGACATC (TGFBR2 mRNA)	AAGGACAUCUUCUCAGACAUC-dTdT GAUGUCUGAGAAGAUGUCCUU-dTdT
Scrambled siRNA	-	GACUUACAACCGUACCGUUAA-dTdT UUAACGGUACGGUUGUAAGUC-dTdT
Fluorescent siRNA	GCAGCACGACTTCTTCAAG (pcDNA3.1-EGFP)	GCAGCACGACUUCUUCAAG-dTdT CUUGAAGAAGUCGUGCUGC-dTdT

2.5 | Plasmid transfection

A549 cells were cultured at a density of 500 × 10^3 cells/well in a six-well culture plate. After 24 hours, at the confluency of 70% to 80%, cells were transfected with either pcDNA3.1 Myc-His A (mock group), full GDF15-pcDNA3.1, mature GDF15-pcDNA3.1, TGFBR2 siRNA, or scramble siRNA. Cells were also cotransfected with full GDF15-pcDNA3.1/TGFBR2 siRNA and mature GDF15-pcDNA3.1/TGFBR2 siRNA. Nontransfected cells are used as control. All transfection experiments were done using lipofectamine LTX withPLUS Reagent (Invitrogen, USA) according to the manufacturer's instructions. To assess transfection efficiency, the cells were transfected with pcDNA3.1-EGFP plasmid and were evaluated 24 and 48 hours after transfection for the expression of EGFP by fluorescence microscopy (Olympus ix71, Japan). Based on EGFP expression, the optimized condition was used for subsequent experiments. To optimize siRNA transfection, various amounts of EGFP-siRNA and pcDNA3.1-EGFP plasmid were cotransfected into A549 cells 48 hours before observation by fluorescence microscopy. Proper concentration of EGFP-siRNA was estimated by measuring the fluorescence intensity of transfected cells. The mRNA expression for GDF15 and TGFBR2 was analysed using real-time-PCR.

2.6 | Real-time PCR

The expression levels of GDF15 and TGFBR2 mRNA were determined using real-time PCR, and the GAPDH and HPRT mRNAs were used as housekeeping genes. The cDNA samples were amplified using SYBRPremix Ex TaqII kit (Takara, Japan) on the StepOnePlus Real-time PCR System (Applied Biosystem, USA) as previously described.[17] Briefly, the reaction mixture consisting of 10 μL of SYBR Premix Ex Taq II, 0.4 μL of ROX reference Dye, 1 μL of cDNA, and 0.25 μM of each primer in a final volume of 20 μL was prepared. The thermal cycling conditions were composed of an initial denaturation step at 95°C for 30 seconds followed by 40 cycles consisting of denaturation at 95°C for 5 seconds and annealing/extension at 60°C for 30 seconds. The experiment was performed in triplicate and repeated three times independently. PCR efficiencies were calculated using LinReg PCR software, and the relative quantification in gene expression was determined using the REST 2009 software. The primers are listed in Table 1.

2.7 | Cell viability assay

The viabilities of A549 cell transfected with full and mature forms of GDF15 and TGFBR2 siRNA were evaluated by using MTT reduction assay. Briefly, A549 cells were seeded on a 96-well plate for 24 hours and transfected with plasmids at confluency of 80%. At desired time after transfection, MTT assay was performed by adding 20 μL of 5 mg/mL MTT reagent (Carl Roth,Germany) to each well and further incubating the cells at 37°C for 4 hours, then the precipitate was solubilized by the addition of 80-μL DMSO (Merck, Germay) per well and shaken for 10 minutes. Absorbance values at 570 nm were then measured with the Anthos 2020 microplate reader. Untransfected

cells were used as a control for 100% cellular viability. All the experiments were done in triplicate and independently repeated three times.

2.8 | Western blotting

Samples were prepared from untransfected or transfected A549 cells with full or mature forms of GDF15. For sample collection, the culture media was collected and centrifuged at 1000 *g* for 5 minutes to separate the detached cells from supernatant. The adhered cells were collected by trypsinization. SDS-PAGE following Western blotting was performed for protein expression. Briefly, cells were lysed with RIPA buffer, and protein concentration was determined by BCA Protein Assay (Pierce, USA). For each sample, 20 to 40-μg total protein was separated on 10% SDS-PAGE and blotted to PVDF membranes. The PVDF membrane was blocked for 2 hours at room temperature with 1% w/v Casein in TBST. The membranes were then probed with primary antibodies overnight at 4°C, followed by horseradish peroxidise conjugated secondary antibodies (1:5000) for 1 hour at room temperature. Finally, blots were developed with BM Chemiluminescence Western Blotting Kit (Roche, Germany) and detected Biomax films (Kodak, USA). The intensity of protein bands was quantified by using ImageJ software. Values for GDF15, caspase 3, caspase 9, and P38 protein were normalized to the β-actin protein, and values for phospho-ERK were normalized to total ERK. Primary antibodies and their dilutions were anti-His6 tag, 1:1000 (Roche, Germany), caspase 3 antibody, 1:1000 (Cell Signaling, USA), caspase 9 antibody, 1:1000 (Cell Signaling, USA), phospho-ERK, 1:1000 (Cell Signaling, USA), total-ERK, 1:1000 (Cell Signaling, USA), and β-actin 1:5000 (Santa Crus, USA).

2.9 | Flow cytometric analysis of apoptosis

The apoptosis was assessed by annexin V/propidium iodide method. A549 cells were transfected either full GDF15-pcDNA3.1, mature GDF15-pcDNA3.1, TGFBR2 siRNA, or cotransfected with full GDF15-pcDNA3.1/TGFBR2 siRNA and mature GDF15-pcDNA3.1/TGFBR2 siRNA and compared with vector (mock) or untransfected A549 cells. The cells were collected 24 hours after and subjected to the apoptosis assay using Annexin V Apoptosis Detection Kit FITC (ebioscience, USA) and evaluated by FACS calibur system (BD, USA). A minimum of 10 000 cells were evaluated and then analysed with flowjo 7.6.1 software.

2.10 | Statistical analysis

The data were analysed using one-way ANOVA followed by Tukey-Kramer post test. The *P* value less than .05 ($P < .05$) was considered significant.

3 | RESULTS

3.1 | Evaluation of expression of GDF15 and silencing of TGFBR2 in A549 cells by real-time PCR

Evaluation of the mRNA expression of full length GDF15 indicated significant increase (>100 fold) in the GDF15 mRNA level in full GDF15 transfected A549 cells compared with un-transfected cells (Figure 1A). To evaluate the efficacy of the TGFBR2 silencing, we checked the TGFBR2 mRNA level in siRNA transfected cells. The results indicated that the TGFBR2 mRNA level was decreased by 97% (mean = 0.027, S.E. range = 0.004-0.153) compared with the control A549 cells (Figure 1B).

3.2 | Expression analysis of full and mature GDF15 in the transfected A549 cells and the supernatants by Western blotting

Because the overexpressed full and mature GDF15 proteins have C-terminal His6-Tag, western blot assay using anti His-Tag antibody was carried out to determine the protein expression of transfected cells and its supernatants. As shown in Figure 1, A549 transfected cells expressed both forms of GDF15 (full and mature, 37.5 and 16 kDa, respectively). The full length GDF15 was expressed in the adherent cells and detached cell (Figure 1C). The mature form was also well expressed in the adherent cells and their supernatant (Figure 1D); these confirmed the proper expression of full and mature GDF15 constructs.

3.3 | The expression of full and mature GDF15 induced cytotoxicity in A549 cells

MTT assay was used to investigate the effect of full and mature GDF15 overexpression on A549 cells viability. Full GDF15 overexpression led to a 41.5% decrease in A549 cell viability at 24 hours and 59% reduction at 48 hours after transfection (Figure 2A). The expression of mature form of GDF15 had no cytotoxicity in A549 cells at 24 hours compared with untransfected cells; however, the cytotoxicity was increased to 51% at 48 hours (Figure 2A). One-way ANOVA showed that full GDF15 transfected cells represented a significant reduction of cell growth rate after 24 and 48 hours (Figure 2A, $P < .001$). The mature GDF15 expression transfected had a significant cytotoxicity only 48 hours after transfection (Figure 2A, $P < .001$).

3.4 | The secreted GDF15 in medium reduced viability in untransfected A549 cells

As a secreted protein, we tested whether the secreted GDF15 from overexpressing cells has any cytotoxicity effect on untransfected A549 cells. The cell culture medium of untransfected A549 cells was replaced by the 24 or 48-hour conditioned medium (24 and 48-hour supernatants) of full and mature GDF15 producing cell, and after 24 hours, the viability was evaluated (Figure 2B). The A549 cell

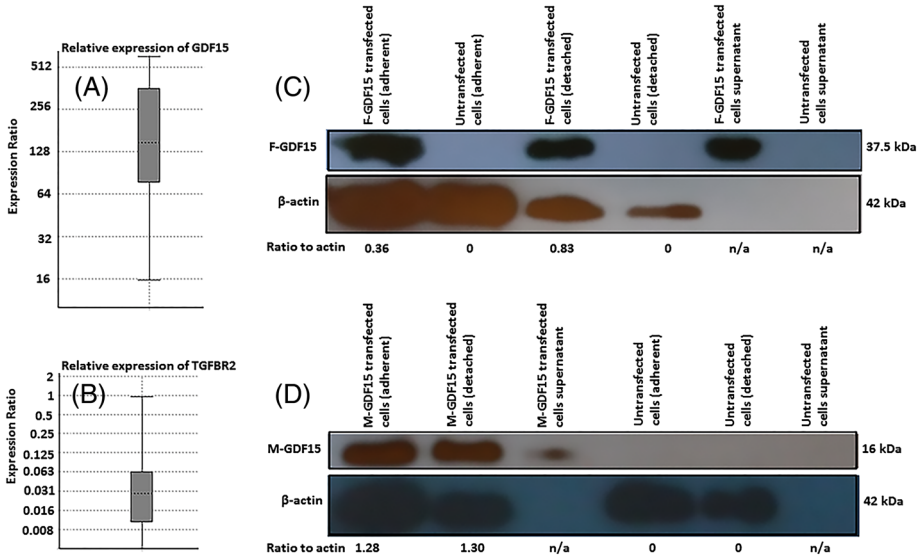

FIGURE 1 Evaluation of expression of GDF15 and TGFBR2 in A549 cells by real-time PCR and Western blotting analysis. A,B, The cells were transfected with full GDF15 expressing construct A, or TGFBR2 specific siRNA B,. Total RNA was extracted and analysed for mRNA expression by RT-qPCR using specific primers. GAPDH and HPRT expression was used as housekeeping genes to normalize the data. PCR efficiencies were calculated using LinReg PCR software, and the relative quantification in gene expression was determined using the REST 2009 software. The values are presented as mean ± S.E. Range of three independent experiments (n = 3). C,D, Cells were transfected with full or mature GDF15 constructs for 24 h, and the adherent or detached cells as well as their supernatants were collected separately. All samples were lysed and evaluated by western blot for the full (C, 37.5 kDa) and mature (D, 16 kDa) forms of GDF15 protein expressions as described in methods and materials

receiving 24-hour supernatant from full length and mature forms of GDF15 expressing cell showed 38% and 35% reduction in cell viability, respectively (Figure 2B). The cells receiving the 48-hour conditioned media also showed significant cytotoxicity as 50% and 47% reduction in cell viability for full and mature forms, respectively (Figure 2B). These results indicated that the secreted GDF15 had cytotoxic effect on untransfected cells.

3.5 | The effect of TGFBR2 silencing on A549 cell viability

In A549 cells, specific silencing of TGFBR2 led to a 38% decrease in A549 cell viability at 24 hours, compared with untransfected or scrambled transfection (Figure 2C, $P < .001$). At 48 hours after silencing, the viability was reduced to 18% reduction compared with control cells (Figure 2C). Statistical analysis indicated that TGFBR2 knocked down significantly reduced the A549 cell viability after 24 and 48 hours ($P < .001$). The viability of A549 cells were reduced to 60% at 24 hours and 80% at 48 hours after siRNA transfection (Figure 2C). There was no significant difference between the cell viability of control and scramble siRNA transfected cells.

3.6 | The TGFBR2 silencing inhibited cytotoxicity induced by GDF15 in A549 cells

To evaluate the role of TGFBR2 on GDF15 effect, we silenced the TGFBR2 in GDF15 transfected A549 cells. As shown in Figure 2D, the knockdown of TGFBR2 completely blocked the cytotoxicity in GDF15 overexpressing cells, and the viability was returned to control level (Figure 2D). To evaluate the effect of secreted GDF15, in cells transfected with TGFBR2 siRNA, the culture medium was replaced with supernatants from full or mature GDF15 producing cells, and the viability was evaluated after 24 hours. Results indicated that the viability of untransfected A549 treated with the GDF15 producing cell 24 and 48-hour supernatants were reduced to 48.7% and 56%, respectively (Figure 2E). The cytotoxicity induced by conditioned media was eliminated in A549 cell lacking TGFBR2 (Figure 2E), suggesting the required role of TGFBR2 in GDF15 cytotoxicity in A549 cells. These data showed that secreted full and mature GDF15 suppressed the cell proliferation in wild-type A549 cells but not in TGFBR2 knocked down cells (Figure 2E). These experiments demonstrated that expression of TGFBR2 was essential for GDF15 induced cytotoxicity. On the other hand, expression or presence of GDF15 inhibited the cytotoxicity induced by lack of TGFBR2. Therefore, one can conclude that GDF15 induces cytotoxicity in the presence of TGFBR2 and may induce survival in the absence of TGFBR. These findings suggest that the TGFBR2 is required for GDF15 induction of cytotoxicity.

3.7 | Expression of full and mature GDF15 protein activates apoptosis via caspase 9 and 3 pathways in wild-type A549 but not in TGFBR2 knocked down cells

To evaluate the cytotoxicity induced by GDF15 in A549 cells, we investigated if cell death/apoptosis was induced by GDF15.

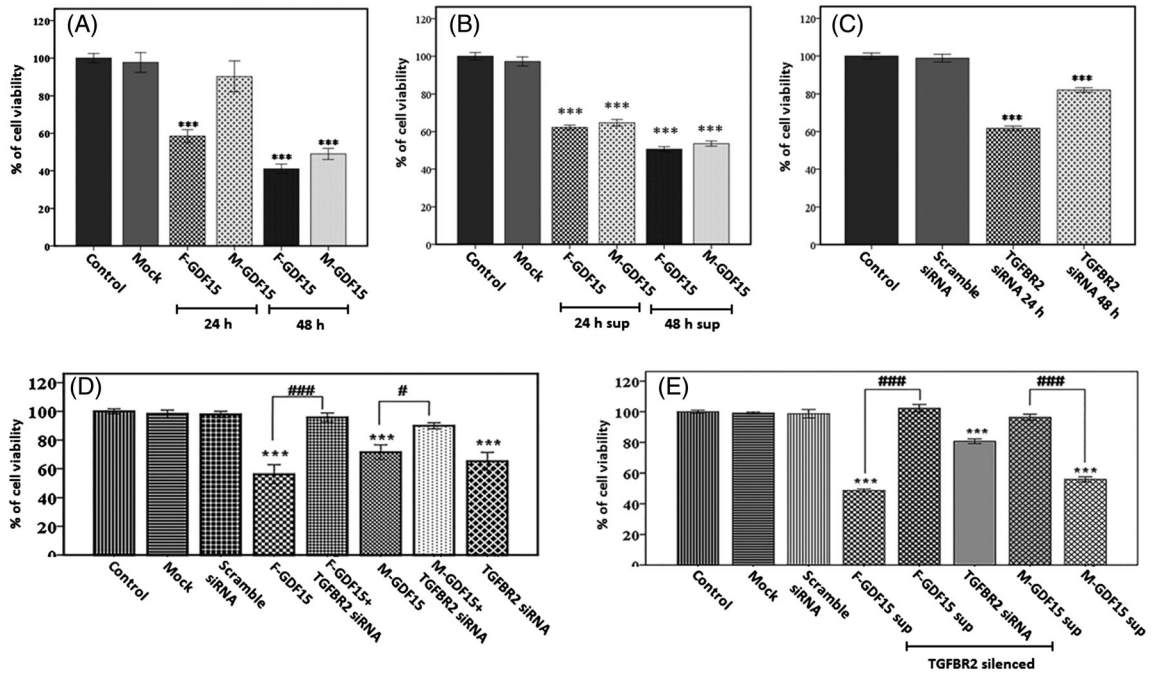

FIGURE 2 Viability assay of GDF15 transfected or TGFBR2 silenced A549 cells using MTT assay. A, The effect of full and mature GDF15 proteins on cell viability in A549 cells. Cells were cultured at 2× 10^4/well in 96 well plates in the DMEM-F12 medium; after 24 h, the cells were transfected with full or mature GDF15 constructs. After 24 and 48 h, cell proliferation was evaluated by MTT assay as described in materials and methods. B, The effect of full and mature GDF15 producing cell supernatant on A549 cell viability. Cells were cultured at 2 × 10^4/well in 96 well plates in the DMEM-F12 medium; after 24 h, the medium was replaced by the conditioned media of full or mature GDF15 producing cell (24 and 48-h supernatant). After 24 h, the cell proliferation was evaluated by MTT assay. C, The effect of TGFBR2 specific silencing on A549 cell viability. Cells were cultured at 2 × 10^4/well in 96 well plates in DMEM-F12 medium; after 24 h, the cells were transfected with TGFBR2-specific siRNA or scrambled siRNA. After 24 and 48 h, cell proliferation was evaluated by MTT assay as described in materials and methods. D, The effect of cotransfection of GDF15 with TGFBR2 siRNA on A549 cell viability: The cells were transfected with either full or mature GDF15 constructs or cotransfected with TGFBR2 specific siRNA, and cell viability was evaluated by MTT assay. E, The effect of full and mature GDF15 producing cell supernatant on TGFBR2 silenced A549 cell viability. Cells were cultured at 2 × 10^4/well in 96 well plates in DMEM-F12 medium and transfected with TGFBR2 siRNA. The culture medium was replaced by full or mature GDF15 producing cell supernatants. After 24 h, the cell proliferation was evaluated by MTT assay. In all the viability assays, the percentage of cell viability was calculated relative to control group. Data are presented as mean ± SE of three independent experiments and analysed by one-way ANOVA followed by a Tukey's post test. (***P < .001 compared with control, ###P < .001, ##P < .01, #P < .05 compared with indicated group). F-GDF15: full GDF15, M- GDF15: mature GDF15, sup: supernatant. Control: Un-transfected A549 cells, mock: empty vector (pcDNA3.1 Myc His A)

Using annexin V/PI flow cytometric analyses, we found that apoptosis was increased to 39.6% and 16.5% in full and mature GDF15 transfected cells, respectively (Figure 3). When TGFBR2 was silenced by siRNA, in A549 cells expressing full or mature forms of GDF15, the apoptosis induced by GDF15 was reduced to control level (Figure 3), suggesting the role of TGFBR2 in GDF15 function. The caspase activations were evaluated to address the mechanism of GDF15 induced apoptosis. As indicated in Figure 4, GDF15 expression increases cleaved caspase 3 and caspase 9 compared with mock (empty vector) transfected cells (Figure 4A,B). TGFBR2 siRNA transfection resulted in no activation of caspase 3 and 9 comparable to controls (Figure 4A,B). These findings confirm the presence of TGFBR2 for the induction of apoptosis by GDF15 in A549 cells. Analyses of capase9 bands indicated that GDF15 expression stimulated caspase 9 cleavage by 8 and 5.7 fold compared with mock transfection for full and mature forms, respectively (Figure 4D). The caspase 3 activation was increased by 4.1 and 2.8 fold increase in full and mature GDF15 transfected A549 cells (Figure 4C). The data also showed that TGFBR2 knocked down resulted in 44% apoptosis in A549 cells (Figure 3). Knockdown of TGFBR2 increased caspase 9 and 3 cleavage by 3 and 1.9 times compared with scrambled control, respectively (Figure 4C,D).

3.8 | ERK1/2 and p38 MAPK phosphorylation was suppressed by GDF15 overexpression in A549 cells but not in TGFBR2 knocked down cells

A549 cells were transiently transfected with full GDF15 and mature GDF15 plasmids or in combination with TGFBR2 siRNA. After 24 hours, cells were lysed and subjected to western blot as described in methods. As shown in Figure 5, expression of full GDF15 or its mature form inhibited phosphorylation of both ERK1/2 (p-ERK 44 kDa and p-ERK 42 kDa) as well as p38 MAPK compared with untransfected control cells. These data demonstrate the role of ERK and p38 pathways in GDF15 induced cytotoxicity. The cells

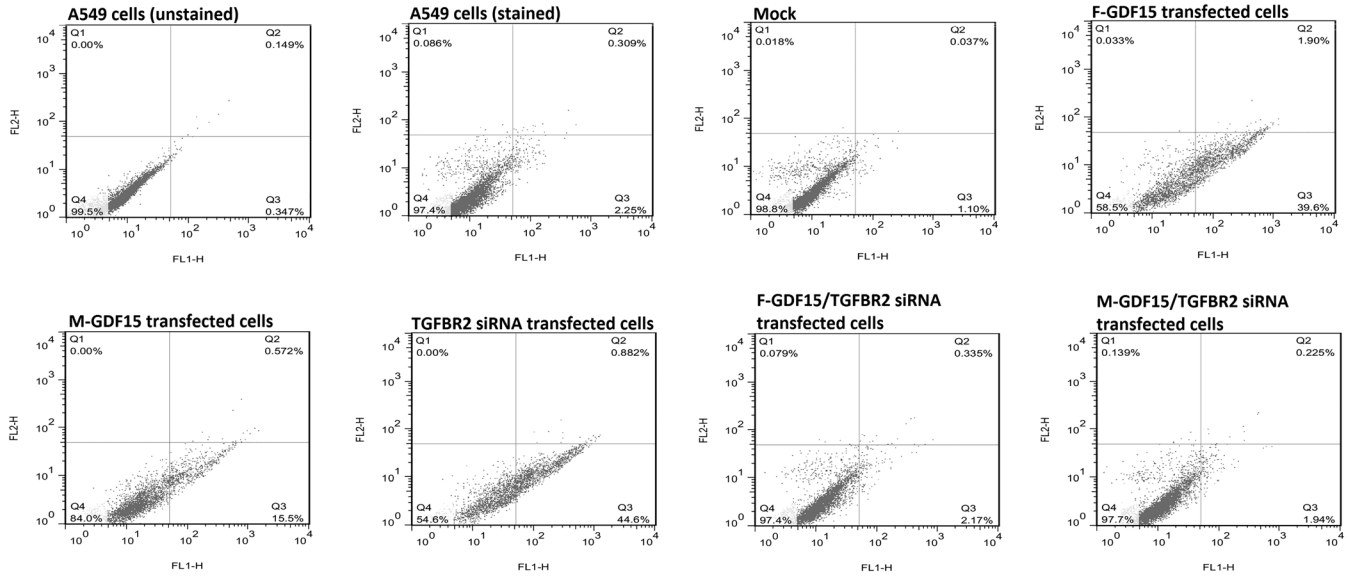

FIGURE 3 The effect of expression of full GDF15, mature GDF15, and co-transfection of TGFBR2 siRNA on apoptosis in A549 cells. Cells were seeded in a six-well plate (5×10^5 cells well) and transfected either with full GDF15, mature GDF15, and TGFBR2 specific siRNA and cotransfection with full GDF15/TGFBR2 siRNA or mature GDF15/TGFBR2 siRNA. The cells were incubated with Annexin V-FITC and PI as described in methods, and the apoptosis was determined using flow cytometry

transfected with TGFBR2 siRNA showed no significant changes in p38 and ERK phosphorylation levels. However, when TGFBR2 was silenced in A549 cells expressing full length or mature GDF15, the inhibitions of ERK1/2 and p38 activation by GDF15 expression were blocked the level of MAPK activations were similar to control (Figure 5). Therefore, knockdown of TGFBR2 eliminated GDF15

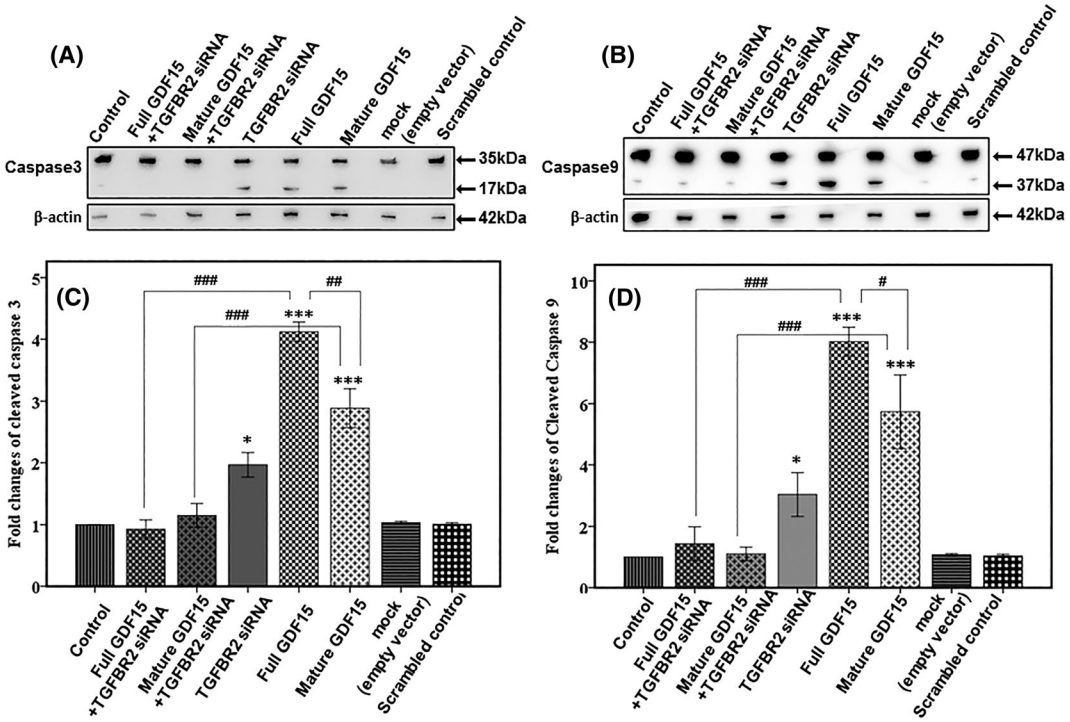

FIGURE 4 The effect of full and mature GDF15 on activation of caspase 3 and 9 in wild-type and TGFBR2 silenced A549 cells. Cells were transfected with either full or mature GDF15 constructs or cotransfected with full GDF15/TGFBR2 siRNA or mature GDF15/TGFBR2 siRNA for 24 h and were lysed. The cleaved caspase 3 (A), and 9 expression (B) were evaluated by western blot as described in methods and materials. The bands of cleaved caspase 3 (C), and caspase 9 (D), proteins were quantified by ImageJ, normalized by caspase3/actin and plotted. The values are presented as mean ± SE of three independent experiments (n = 3) and analysed by one-way ANOVA followed by a Tukey's post test (*$P < .05$; **$P < .01$;***$P < .001$ versus control and #$P < .05$; ##$P < .01$; ###$P < .001$ compared with indicated groups)

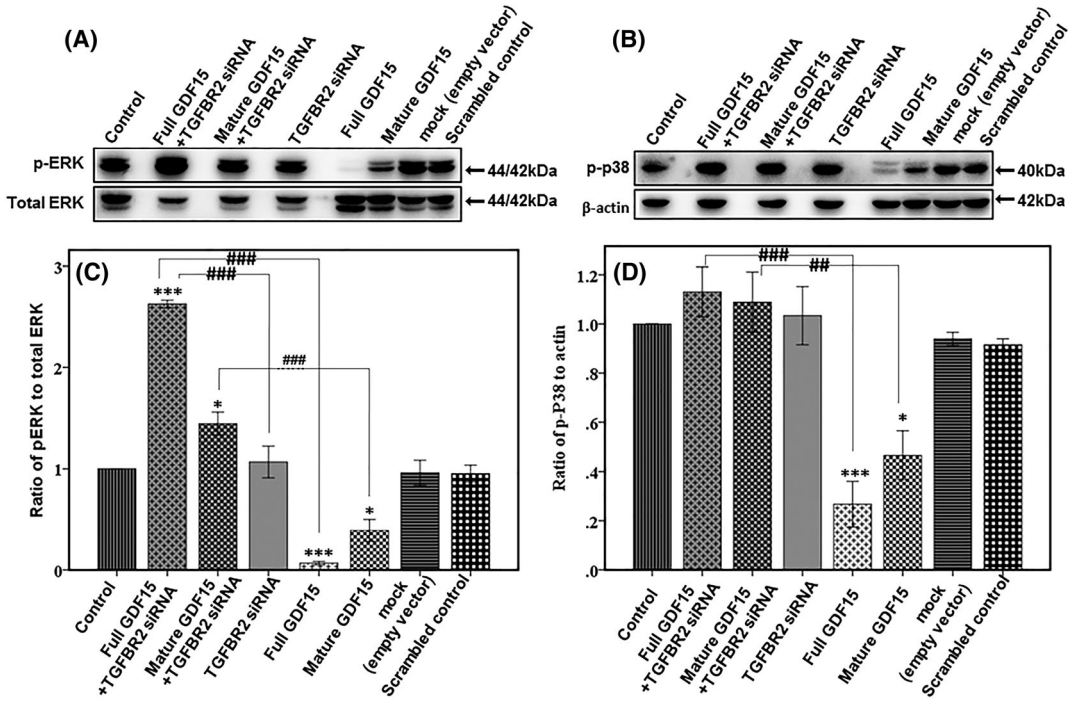

FIGURE 5 The effect of full and mature GDF15 on phosphorylation of ERK1/2 and p38 in wild-type and TGFBR2 silenced A549 cells. Cells were transfected with either full or mature GDF15 constructs or cotransfected with full GDF15/TGFBR2 siRNA or mature GDF15/TGFBR2 siRNA for 24 h, and the cell lysates were subjected to SDS-PAGE. The p-ERK (A), and p-P38 (B), levels were evaluated by western blot as described in methods and materials. The bands of p-ERK (C), and p-P38 (D), protein were quantified by ImageJ, normalized by total ERK and β-actin, respectively, and plotted. The values are presented as mean ± SE of three independent experiments (n = 3) and analysed by one-way ANOVA followed by a Tukey's post test (**P* < .05; ***P* < .01;****P* < .001 versus control and #*P* < .05; ##*P* < .01; ###*P* < .001 compared with indicated groups)

inhibition of ERK1/2 and p38 phosphorylation. These findings suggest the role of TGFBR2 in GDF15 inhibition of ERK1/2 and p38 activation.

4 | DISCUSSION

The roles of GDF15 have been reported in various cellular functions including the inhibition and promotion of cancer tissue.[18] The mechanisms of the dual functions during carcinogenesis are important for proper approach in cancer therapy. Moreover, there are reports indicating the role of TGFBR2 in the signalling of GDF15[13] Besides, the GDF15 protein is expressed as full length and secreted as mature form; thus, one can consider diverse effects for these forms. Therefore, in this study, we have evaluated the role of full length and mature form of GDF15 in induction of apoptosis with regard to the expression of TGFBR2.

The overexpression of both full and mature GDF15 reduced the viability of A549 (Figure 2A). The effect of full length was from 24 to 48 hours; however, the mature form had shown cytotoxicity only after 48 hours. These finding indicated that full and mature GDF15 exert antiproliferative effect on A549 cells. Furthermore, the GDF15 expression induced apoptosis and cell death in A549 cells (Figures 3 and 4) confirming the antiproliferative effect of GDF15. These results are in agreement with previous findings which showed that overexpression of GDF15 reduced cell growth rate and induced apoptosis in different types of cells including A549 cells.[19-21] In addition, GDF15 has proapoptotic and antitumorigenic activities and exerts tumour suppressor role in various cancers.[22,23] Although both forms have inhibited the viability of the cell, the inhibitory effect of full GDF15 is in shorter time than mature GDF15 in A549 cells; this can be because of the presence of the N-terminal domain in full GDF15 which affects protein folding and stability and influences secretion efficiency and consequently improves its performance.[24] In this regard, treatment of untransfected A549 with the conditioned media (supernatant of both full and mature GDF15 transfected cells, Figure 2B) has had cytotoxic effect at 24 and 48 hours, suggesting the effect of mature form in induction of growth inhibition.

When the TGFBR2 was silenced with siRNA in A549 cells, the cell viability was reduced (Figure 2C). These data are in line with the results of previous studies indicating siRNA knockdown of TGFBR2 reduces cell viability and induces apoptosis.[25,26] However, other studies showed that high expression levels of TGFBR2 can induce apoptosis in cells.[27,28] These findings suggest that TGFBR2 is critical for cell survival and any abnormal changes in TGFBR2 level can induce cell death.

Interestingly, we found that silencing TGFBR2 in A549 cells completely blocked the cytotoxicity induced by full or mature GDF15, suggesting a major role for TGFBR2 in GDF15 induced cytotoxicity (Figure 2D). Similarly, in A549 cells with silenced TGFBR2 expression, the secreted GDF15 in conditioned media could not

induce cytotoxicity, and its effect was blocked in the absence of TGFBR2 (Figure 2E). These findings point toward the importance of TGFBR2 for the cytotoxic effect of GDF15 in A549 cell proliferation. In this regard, the overexpression of GDF15 (full or mature forms) in A549 cell induced activation of caspase 9 and 3 as well as apoptosis (Figures 3 and 4). The activations of both caspases and induction of apoptosis by GDF15 were completely blocked by silencing of TGFBR2 in A549 cells although silencing TGFBR2 activated caspases and cell death to lesser extent. All these findings confirm the role of TGFBR2 in induction of cell death in A549 cells by GDF15.

Findings from other groups have indirectly reported a role for TGFBR2 in GDF15 effect in cancer cells. It has been shown that GDF15 induces apoptosis in cells expressing TGFBR2 including A549, MCF-7, DU-145, and LNCaP cells.[21,29,30] However, in OSCC cells which have low expression of TGFBR2, GDF15 lacks cell death/apoptotic effect and induces proliferation.[11,12] Moreover, in prostate cancer cells, downregulation of TGFBR2 via microRNA-21 resulted in lack of TGFβ growth inhibition.[31] Therefore, one can consider a potential role for TGFBR2 in GDF15 induced cell death.

In our study we have shown that silencing TGFBR2 completely blocks GDF15 induced cytotoxicity in A549 cell. Furthermore, silencing TGFBR2 inhibited GDF15 activation of caspase 9 and caspase 3 in these cells. These results are in agreement with the results of other studies.[32-34] Suppression of TGFBR2 by specific siRNA induced apoptosis via increasing cleavage of caspase 9 and 3 in A549 cells; this data is in agreement with previous findings[25,26] and is in contradiction to other studies.[35-37] Thus, the presence of TGFBR2 can mediate GDF15 inhibitory effect on cell proliferation. This further proves that TGFBR2 can act as a receptor for GDF15 induced cell death.

The dual role of GDF15 as increase in cell proliferation or induction of cell death may relate to expression status of TGFBR2 and can be related to EGFR. It has been shown that in the absence of TGFBR2, GDF15 increases A549 cell proliferation rate through another receptor maybe via EGFR.[38] It has been reported that activation of AKT and ERK1/2 by GDF15 via ErbB2 promotes proliferation of breast, cervical, and gastric cancer cells[39,40] although the level of TGFBR2 has not been reported in these cells. It is also reported that the effect of GDF15 on increasing ERK and AKT phosphorylation mediate via GFRAL and its co-receptor RET.[2] On the other hand, in cells with high level of TGFBR2, the growth inhibition has been observed by GDF15.[21,29,30] In addition, it has been shown that TGFBR2 can have diverse function during carcinogenesis and can undergo various types of inactivating mutations.[16]

We found that overexpression of GDF15 decreased phosphorylation of p38 MAPK and extracellular signal-regulated kinases (ERK1/2) in A549 cells (Figure 5). These findings are similar to Cekanovaet al. that overexpression of GDF15 has resulted in a significant reduction in phosphorylated p38 MAPK in A549 cells; however, they have reported no effect on the phosphorylation of ERK1/2.[34] The effects of GDF15 on p38 and ERK1/2 MAPK may be cell context dependent. In some ovarian cancer cells, GDF15 induces cell proliferation via p38 and ERK1/2 activation, and in HER2 overexpressing breast cancer cells, GDF15 contributes to proliferation and invasiveness via p38 activation.[41,42] Interestingly, TGFBR2 siRNA blocked the GDF15 effect on p38 and ERK1/2 phosphorylation (Figure 5), further suggesting the role of TGFBR2 in GDF15 signalling in A549 cells.

Our data showed that knockdown of TGFBR2 did not significantly change ERK1/2 and p38 MAPK phosphorylation in A549 cells. However, it has been previously reported that MAPK-ERK signalling pathway activation correlates with TGFBR2 expression.[27] Besides, in MCF7 cells, siRNA-mediated TGFBR2 knockdown has decreased ERK phosphorylation with no changes in p38 MAPK phosphorylation.[43]

The underlying molecular mechanism of GDF15 functions as an antitumour or tumour promoting agent in cancer cell is incomplete. In the present study, our findings suggest that the effect of GDF15 protein on A549 cells depends on the TGFBR2 status and induces apoptosis in the presence of TGFBR2. Thus, the effect of GDF15 depends on the TGFBR2 status. This is probably one of the reasons which GDF15 shows equivocal activities during carcinogenesis in many cancers with elevated serum levels of GDF15.[5,44,45] Therefore, serum GDF15 can play a role to inhibit or promote the growth of cancer cells depending on the TGFBR2 status. The issue becomes important because NSAIDs increase the expression of GDF15.[3,46] So, administration of NSAIDs for patients with malignancies prone to TGFBR2 mutation must be carefully considered, because there is a possible role in the tumour growth. Increasing amount of GDF15 in the context of late stage tumours can inhibit tumour-specific immune cells and suppress immune responses against tumour cells[47] and can promote proliferation of tumour cells in the late stage of malignancies.

5 | CONCLUSION

Taken together, our results show that the GDF15 (as full or mature forms) can induce cell death and cytotoxicity. The effect of GDF15 is mediated via activation of p38 and ERK1/2 pathways and depends on the presence of TGFBR2. In lack of TGFBR2 expression, GDF15 has no apoptotic effect in A549 cells. The dual effects of GDF15 may explain the role of the protein during carcinogenesis and inhibit tumour cell growth based on the TGFBR2 function. Understanding the mechanisms of GDF15 dual effect and function provides information for effective cancer chemotherapy.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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DATA AVAILABILITY STATEMENT

There is no other data available for this article.[48] All the data have been used in the figures.

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