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Basic Study

Downregulation of *TNFR2* decreases survival gene expression, promotes apoptosis and affects the cell cycle of gastric cancer cells

Ana Flávia Teixeira Rossi, Fernanda da Silva Manoel-Caetano, Joice Matos Biselli, Ágata Silva Cabral, Marília de Freitas Calmon Saiki, Marcelo Lima Ribeiro, Ana Elizabete Silva

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Abstract

BACKGROUND

Chronic inflammation due to *Helicobacter pylori* (*H. pylori*) infection promotes gastric carcinogenesis. Tumour necrosis factor- α (TNF- α), a key mediator of inflammation, induces cell survival or apoptosis by binding to two receptors (TNFR1 and TNFR2). TNFR1 can induce both survival and apoptosis, while TNFR2 results only in cell survival. The dysregulation of these processes may contribute to carcinogenesis.

AIM

To evaluate the effects of TNFR1 and TNFR2 downregulation in AGS cells treated with *H. pylori* extract on the TNF- α pathway.

METHODS

AGS cell lines containing TNFR1 and TNFR2 receptors downregulated by specific shRNAs and nonsilenced AGS cells were treated with *H. pylori* extract for 6 h. Subsequently, quantitative polymerase chain reaction with TaqMan® assays was used for the relative quantification of the mRNAs (*TNFA*, *TNFR1*, *TNFR2*, *TRADD*, *TRAF2*, *CFLIP*, *NFKB1*, *NFKB2*, *CASP8*, *CASP3*) and miRNAs (miR-19a, miR-34a, miR-103a, miR-130a, miR-181c) related to the TNF- α signalling pathway. Flow cytometry was employed for cell cycle analysis and apoptosis assays.

RESULTS

In nonsilenced AGS cells, *H. pylori* extract treatment increased the expression of genes involved in cell survival and inhibited both apoptosis (*NFKB1*, *NFKB2* and *CFLIP*) and the *TNFR1* receptor. TNFR1 downregulation significantly decreased

the expression of the *TRADD* and *CFLIP* genes, although no change was observed in the cellular process or miRNA expression. In contrast, *TNFR2* downregulation decreased the expression of the *TRADD* and *TRAF2* genes, which are both important downstream mediators of the *TNFR1*-mediated pathway, as well as that of the *NFKB1* and *CFLIP* genes, while upregulating the expression of miR-19a and miR-34a. Consequently, a reduction in the number of cells in the G0/G1 phase and an increase in the number of cells in the S phase were observed, as well as the promotion of early apoptosis.

CONCLUSION

Our findings mainly highlight the important role of *TNFR2* in the TNF- α pathway in gastric cancer, indicating that silencing it can reduce the expression of survival and anti-apoptotic genes.

Key Words: Gastric cancer; *Helicobacter pylori*; Tumour necrosis factor- α signalling pathway; *TNFR1*; *TNFR2*; miRNAs

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Core Tip: This study demonstrated for the first time the effect of *TNFR1* and *TNFR2* downregulation on an AGS cell line treated with *Helicobacter pylori* extract. Although *TNFR1* downregulation did not promote significant changes in the expression of mRNA and miRNAs of the tumour necrosis factor- α (TNF- α) signalling pathway, *TNFR2* downregulation promoted important changes in the signalling mediators evaluated. We observed a reduction in the expression of cell survival and anti-apoptotic genes and an increase in the expression of miR-19a and miR-34a, which affected cell cycle kinetics and contributed to early apoptosis. Thus, our findings highlight the important role of *TNFR2* in the TNF- α signalling pathway in gastric carcinogenesis.

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INTRODUCTION

Gastric cancer (GC) has high rates of incidence and mortality worldwide, especially in Eastern Asia, Eastern Europe and Latin America[1]. In Brazil, it is the fourth most common cancer in men and the sixth most common in women[2]. *Helicobacter pylori* (*H. pylori*) is the main risk factor for the development of gastric neoplasms, since it is responsible for chronic inflammation in the gastric mucosa and for the GC progression cascade[3]. Consequently, it can trigger the host's immune response, which leads to changes in the expression of genes related to inflammation, cell kinetic regulation and miRNAs [4-6]. After infection by this bacterium, tumour necrosis factor- α (TNF- α) stands out among the mediators of inflammation and is considered to be a key mediator linking inflammation and cancer[7,8].

TNF- α is a pleiotropic proinflammatory cytokine that is important in the signalling and regulation of multiple cellular responses and processes, such as the production of other inflammatory cytokines, and cell communication, survival, proliferation and apoptosis[9]. These different functions are accomplished due to the ability of TNF- α to bind to *TNFR1* (TNFRSF1A) or *TNFR2* (TNFRSF1B) receptors, thus resulting in different cellular processes. Both receptors are transmembrane proteins, and although they are largely similar in terms of extracellular structure, their intracellular domains are different, and thus dictate the cellular fate for either survival or death. Though only *TNFR1* has a death domain, the TNF- α signalling pathway triggered by this receptor is able to induce both cell survival and apoptosis, while *TNFR2* results only in cell survival[10,11]. The signalling cascade after TNF- α /*TNFR1* binding that results in cell survival starts with the recruitment of *TRADD* and is mediated by activation of nuclear factor-kappaB (NF- κ B) and transcription of pro-survival and anti-apoptotic genes, such as cellular inhibitor of apoptosis proteins (cIAP), *TRAF2* and *cFLIP*, and of inflammatory cytokines. However, this signal complex is transient; TNF- α rapidly dissociates from *TNFR1* and binds to the Fas-associated death domain protein to form another signal complex, which coordinates downstream signalling of the caspase cascade and apoptosis. Conversely, as *TNFR2* does not have a death domain, it induces long-lasting NF- κ B activation by recruiting existing cytoplasmic *TRAF-2*/cIAP-1/cIAP-2 complexes that can inhibit pro-apoptotic factors and maintain cell survival and proliferation[9-12].

Regulation of signal transduction triggered by $TNF-\alpha$ requires a constant balance between the opposing functions of cell survival and cell death to maintain homeostasis. Thus, an imbalance in these processes due to changes in the expression of receptors, downstream genes, ligands and pro-/anti-apoptotic mediators may support the tumorigenic process[13]. A recent study by our research group showed dysregulation in the $TNF-\alpha$ signalling pathway in GC samples with an upregulation of cell survival-related genes and of *TNFR2* expression, thus suggesting a prominent protumor role by $TNF-\alpha$ /*TNFR2* binding in gastric neoplasm[14]. Furthermore, we showed through a miRNA:mRNA interaction network that this signalling pathway can be regulated by miRNAs. In addition, *H. pylori* infection was also associated with an increased expression of *TNF-\alpha* mRNA and protein, and dysregulated miRNA expression in chronic gastritis patients. The expression pattern of these genes/miRNAs was normalized after treatment to eradicate bacteria, indicating that this pathogen influences the host's inflammatory response in part by its action on miRNAs[6].

In accordance with our previous results, we thought it was important to evaluate the effect of silencing *TNFR1* and *TNFR2* receptors in an AGS gastric cell line after treatment with an *H. pylori* extract on *TNF-\alpha* mRNA expression and on downstream genes related to its signalling pathway (*TRADD*, *TRAF2*, *CFLIP*, *NFKB1*, *NFKB2*, *CASP8* and *CASP3*). In addition, we also investigated the same miRNAs previously studied (miR-19a, miR-34a, miR-103a, miR-130a and miR-181c)[14], as well as the cell cycle and apoptosis. Overall, our results highlight the main role of the *TNFR2* in $TNF-\alpha$ signalling in an AGS cell line, while treatment with *H. pylori* extract induces prosurvival gene expression mainly through *TNFR1*.

MATERIALS AND METHODS

Cell culture

AGS GC cells from the Cell Bank of Rio de Janeiro, Brazil (BCRJ code 0311) were incubated at 37 °C and 5% CO₂ in HAM-F10 medium (Cultilab, SP, Brazil) supplemented with 10% foetal bovine serum and 1× antibiotic/antimycotic (Gibco, Invitrogen Life Technologies, Carlsbad, CA, United States). The culture medium was replaced every two to three days. The HGC-27 GC cell line, provided by Dr Marcelo Lima Ribeiro (São Francisco University-USF, SP, Brazil), was also used in early experiments as an alternative line for follow-up experiments, and 293T cells of human embryonic kidney were provided by Dr Luisa Lina Villa (University of São Paulo-USP, SP, Brazil) for transfection experiments. The cells were maintained under the same conditions as the AGS cell line except for the culture medium, which was Dulbecco's modified Eagle medium (Gibco, Invitrogen Life Technologies, Carlsbad, CA, United States). Stable shRNA-expressing cell lines with reduced expression of *TNFR1* (called sh*TNFR1*) and *TNFR2* (called sh*TNFR2*) were kept in a similar culture medium to the nonsilenced AGS cells, and further supplemented with 0.5 µg/mL puromycin or 200 µg/mL G418, respectively.

Treatment with *H. pylori* extract

The previously described *H. pylori* Tox+ strain (cagA+/vacA s1m1) was grown in a selective medium (pylori-Gelose; BioMérieux, Marcy-l'Étoile, France) at 37 °C under microaerophilic conditions[15]. This strain is not resistant to any antibiotic used to treat *H. pylori*. *H. pylori* extract was prepared according to the protocol described by Li *et al*[16]. In brief, the *H. pylori* Tox+ strain was harvested and suspended in distilled water at a concentration of 2×10^8 CFU/mL. Next, the suspension was incubated at room temperature for 40 min and centrifuged at 20000 g for 20 min. The supernatant was filtered using a 0.2 µm filter and stored at -20 °C until use. The extract was tested several times (4, 6 and 24 h) using differing *H. pylori* extract volumes (50, 100, 150 and 200 µL) to verify the best experimental conditions. Then, 2×10^5 nonsilenced AGS cells and AGS cells with downregulation of *TNFR1* (sh*TNFR1*) and *TNFR2* (sh*TNFR2*) were seeded in 12-well plates. After 48 h, the medium was replaced with 500 µL of HAM-F10 containing 30% v/v *H. pylori* extract or the same proportion of water (control). Cells were incubated with *H. pylori* extract for 6 h in an incubator at 37 °C. For all experiments, three temporally independent events were performed.

Cell viability assay

MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) assays (Sigma-Aldrich, St. Louis, MO, United States) were employed to evaluate the viability of silenced and nonsilenced AGS cells after different treatment conditions with *H. pylori* extract (times and *H. pylori* extract volumes). In summary, the culture medium was removed, and 300 µL of fresh medium containing 1 mg/mL MTT reagent was added to each well. After 30 min of incubation at 37 °C, the medium with MTT reagent was replaced by the same volume of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, United States). The absorbance at 540 nm was measured using a FLUOstar Omega spectrophotometer (BMG Labtech, Ortenberg, Germany).

Transfection with shRNA

MISSION® Lentiviral Transduction Particles (Cat. SHCLNV, Sigma-Aldrich, St. Louis, MO, United States) were used to integrate shRNA into the genome of AGS cells to knockdown *TNFR1* and *TNFR2* expression. Verified viral vectors were purchased from Sigma-Aldrich, and the standard manufacturer's protocol was followed to generate the cell line. Stably transfected clones were selected by growing the cells in the presence of puromycin or G418, which acted as selection pressure (for *TNFR1*: 1 µg/mL puromycin and for *TNFR2*: 400 µg/mL G418). Different plasmids containing shRNAs were used to generate clones, of which the one showing the best knockdown efficiency was used for all experiments. The sequence of the shRNA in the construct was CTTGAAGGAACTACTACTAAG for *TNFR1* and GCCGGCTCAGAGAATACTATG for *TNFR2*. *TNFR1* and *TNFR2* levels were assessed by quantitative polymerase chain reaction (PCR) and Western blotting to verify the knockdown. Similar transfections were carried out with an empty vector (Sigma-Aldrich, St. Louis, MO, United States), which served as the transfection control.

Western blot analysis

Protein extraction was performed by lysis of nonsilenced AGS cells and AGS cells containing a shRNA or an empty vector with CelLytic™ MT Cell Lysis Reagent (Sigma-Aldrich, St. Louis, MO, United States). The lysis reaction was centrifuged at 12000 g for 10 min after a 15 min incubation. The protein concentration was determined using a Pierce™ BCA Protein Assay kit (Thermo Scientific, Massachusetts, United States) according to the manufacturer's protocol. Thirty micrograms of protein was separated on an 8%-12% sodium dodecyl sulphate-polyacrylamide gel by electrophoresis (120 min) and then transferred to PVDF or nitrocellulose membranes (Millipore Corporation, Burlington, Massachusetts, EUA) using an Electrotransfer TE70XP system (Hoefer) for 80 min. Membranes were blocked with 5% nonfat dry milk for 60 min and were then incubated overnight at 4 °C with the following primary antibodies: Anti-*TNFR1* (dilution 1:500) (Cell Signalling, Massachusetts, United States), anti-*TNFR2* (dilution 1:5000) (Abcam Cambridge, United Kingdom) and anti-GAPDH (dilution 1:30000) (Abcam, Cambridge, United Kingdom). After being washed, the membranes were incubated at room temperature under stirring with horseradish peroxidase-conjugated goat anti-rabbit (dilution 1:2000) secondary antibodies (Abcam, Cambridge, United Kingdom). Bands were revealed by enhanced chemiluminescence, visualized in a ChemiDoc™ Imaging System (BioRad, Hercules, California, United States) and quantified using Image Lab 6.0 Software (BioRad, Hercules, California, United States).

Quantification of mRNA and miRNA expression by RT-qPCR

After 6 h of incubation, the medium containing *H. pylori* extract or water was removed, and total RNA was extracted from AGS cells with a miRNeasy Micro Kit (Qiagen, Valencia, CA, United States) according to the manufacturer's protocol. Complementary DNA (cDNA) for mRNA and miRNA was synthesized using a High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, United States) and a TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, United States), respectively. Quantitative PCR (qPCR) was performed with TaqMan® assays (Applied Biosystems, California, United States) for the genes *TNFA* (Hs01113624_g1), *TNFR1* (*TNFRSF1A*) (Hs01042313_m1), *TNFR2* (*TNFRSF1B*) (Hs00961749_m1), *TRADD* (Hs00182558_m1), *TRAF2* (Hs00184192_m1), *CFLIP* (*CFLAR*) (Hs00153439_m1), *NFKB1* (Hs 00765730_m1), *NFKB2* (Hs01028901_g1), *CASP8* (Hs01116281_m1) and *CASP3* (Hs00234387_m1) and for the target miRNAs hsa-miR-19a-3p (MIMAT0000073; ID 000395), hsa-miR-34a-3p (MIMAT0004557; ID 002316), hsa-miR-103a-3p (MIMAT0000101; ID 000439), hsa-miR-130a-3p (MIMAT0000425; ID 000454) and hsa-miR-181c-5p (MIMAT0000258; ID 000482) (Applied Biosystems, California, United States), as described in our previous study [14]. *ACTB* (Catalogue#: 4352935E) and *GAPDH* (Catalogue#: 4352934E) genes were used for normalization of mRNA quantification, while endogenous RNU6B (ID 001093) and RNU48 (ID 001006) levels were used for miRNAs. All reactions were performed in triplicate. Relative quantification (RQ) of mRNA and miRNA expression was calculated by the $2^{(-\Delta\Delta Ct)}$ method [17], and nonsilenced AGS without *H. pylori* extract treatment was used as a calibrator (AGS-C). RQ values are expressed as the mean ± SD of gene and miRNA expression for all experimental groups in relation to nonsilenced and untreated AGS, with RQ = 1.

Cell cycle analysis

The cell distribution at different phases of the cell cycle was estimated by measuring the cellular DNA content using flow cytometry. After incubation with *H. pylori* extract or water, nonsilenced AGS, sh*TNFR1* and sh*TNFR2* cell lines were harvested with trypsin and fixed in 70% ethanol at 4 °C for at least 24 h. Subsequently, the cells were washed, centrifuged and incubated with 200 µL of Guava® Cell Cycle Reagent (Merck Millipore, Burlington, Massachusetts, United States) for 30 min in the dark. Cell cycle distribution was measured by a Guava® EasyCyte Flow Cytometer and analysed with Guava® InCyte software (Merck Millipore, Burlington, Massachusetts, United States).

Apoptosis analysis

Apoptotic cell death was also measured by flow cytometry using a fluorescein isothiocyanate (FITC)

Annexin V Apoptosis Detection Kit (BD Pharmingen™; BD Biosciences, Franklin Lakes, NJ, United States) according to the modified manufacturer's protocol. After treatment, adherent cells were harvested with Accutase® (Sigma-Aldrich, St. Louis, MO, United States) since this solution avoids membrane damage[18]. After this step, the cells were washed with cold phosphate-buffered saline, centrifuged, resuspended in 1× binding buffer and stained with 5 µL of Annexin V-FITC and 5 µL of propidium iodide (PI, 50 µg/mL) for 15 min at room temperature in the dark. The labelling of cells was evaluated using a flow cytometer and was analysed with Guava® InCyte software. From the scatter diagram and quadrant plotting, the results are presented as follows: Living cells (FITC-/PI-) located in the lower left quadrant, early apoptotic cells (FITC+/PI-) in the lower right quadrant and late apoptotic cells (FITC+/PI+) in the upper right quadrant.

Statistical analysis

Statistical analysis of the data was performed in GraphPad Prism Software version 6.01 using two-way ANOVA with Bonferroni post hoc test. The results are expressed as the mean ± SD from three experiments conducted independently. A $P < 0.05$ was considered statistically significant.

RESULTS

The AGS cell line treated with *H. pylori* extract expresses *TNFA*, *TNFR1*, and *TNFR2* receptors

Initially, both AGS and HGC-27 cell lines were cultured to evaluate the mRNA expression levels of *TNFA*, *TNFR1* and *TNFR2* receptors by qPCR. The analyses showed that both cell lines expressed *TNFA* and *TNFR1*, but *TNFR2* was expressed at lower levels in HGC-27 cells than it was in AGS cells (Supplementary Figure 1). The AGS cell line was chosen for additional experiments since it was not reasonable to use HGC-27 in the *TNFR2* silencing experiments.

To standardize treatment conditions, several volumes of *H. pylori* extract (50, 100, 150 and 200 µL) were tested at different incubation times (4, 6 and 24 h) in the AGS cell line to establish the best conditions for inducing *TNFA* expression without reducing cell viability. The best results were obtained with an *H. pylori* extract concentration of 30% v/v (150 µL) at 6 h of incubation (Supplementary Figures 2 and 3). Under these conditions, there was an increase in *TNFA* expression (RQ = 3.59) without an impact on cell viability (98.9%).

The effect of *TNFR1* and *TNFR2* downregulation and treatment with *H. pylori* extract on *TNF-α* signalling downstream gene expression

The effect of *TNFR1* and *TNFR2* downregulation on GC cells was evaluated after transfection of the AGS cell line with two specific shRNAs targeting the genes, *TNFR1* (called sh*TNFR1*) and *TNFR2* (called sh*TNFR2*), followed by antibiotic selection. RT-qPCR and Western blotting were used to evaluate the efficiency of silencing. Stable lines with the shRNAs exhibited a 59% reduction in *TNFR1* and a 63% reduction in *TNFR2* protein expression (Figure 1).

Initially, we evaluated the effect of treatment with *H. pylori* extract on the expression of genes of the *TNF-α* signalling pathway in nonsilenced AGS cells. After *H. pylori* extract treatment (AGS-*H. pylori* extract), nonsilenced AGS cells showed significantly upregulated mRNA expression of *TNFR1* and of anti-apoptotic and cell proliferation genes, such as *CFLIP*, *NFKB1* and *NFKB2* (Supplementary Figure 4A), compared to the control (AGS-C). In addition, *H. pylori* extract treatment increased the mRNA expression of *NFKB1* and *NFKB2* in a *TNFR1*-downregulated cell line (sh*TNFR1*-*H. pylori* extract), whereas the expression of *TNFR2* and *TRADD* was reduced (Supplementary Figure 4B). On the other hand, the expression of the evaluated genes was not significantly changed by *H. pylori* extract treatment in the sh*TNFR2* cell line (Supplementary Figure 4C).

With regard to the influence of *TNFR1* and *TNFR2* downregulation on the expression of *TNF-α* signalling genes (Figure 2), a significantly downregulated mRNA expression of *TNFR1* (RQ = 0.83 and 1.03, respectively), *TRADD* (RQ = 0.60 and 0.41, respectively) and *CFLIP* (RQ = 1.25 and 1.36, respectively) was observed in sh*TNFR1*-*H. pylori* extract and sh*TNFR2*-*H. pylori* extract cells compared to that in AGS-*H. pylori* extract cells (RQ = 1.73, 0.98 and 2.09, respectively) (Figure 2B, D and F). Furthermore, *TNFR2* (RQ = 0.27, $P < 0.05$), *TRAF2* (RQ = 0.68, $P < 0.05$) and *NFKB1* (RQ = 1.67, $P < 0.05$) were reduced in sh*TNFR2*-*H. pylori* extract cells compared to AGS-*H. pylori* extract cells (RQ = 0.86, 1.29 and 2.39, respectively) (Figure 2C, E and I). When we compared the nonsilenced and silenced AGS cell lines without *H. pylori* extract treatment (control-C), *TNFR2* mRNA expression was increased in sh*TNFR1*-C (RQ = 1.49) compared to AGS-C (RQ = 1.00) and reduced in sh*TNFR2*-C (RQ = 0.50) compared to sh*TNFR1*-C and AGS-C. Likewise, *TRADD* mRNA expression was downregulated in sh*TNFR2*-C (RQ = 0.35) compared to the other control groups (AGS-C and sh*TNFR1*-C). There was no significant change in *TNFA*, *CASP3*, *CASP8* and *NFKB2* mRNA expression.

In general, the downregulation of *TNFR1* and *TNFR2* significantly influenced the mRNA expression of *TRADD* ($P < 0.001$) and *TRAF2* ($P < 0.01$), whereas the expression of *TNFR1*, *TNFR2* and *CFLIP* was also affected by treatment with *H. pylori* extract ($P < 0.01$ for all). In contrast, only the *H. pylori* extract

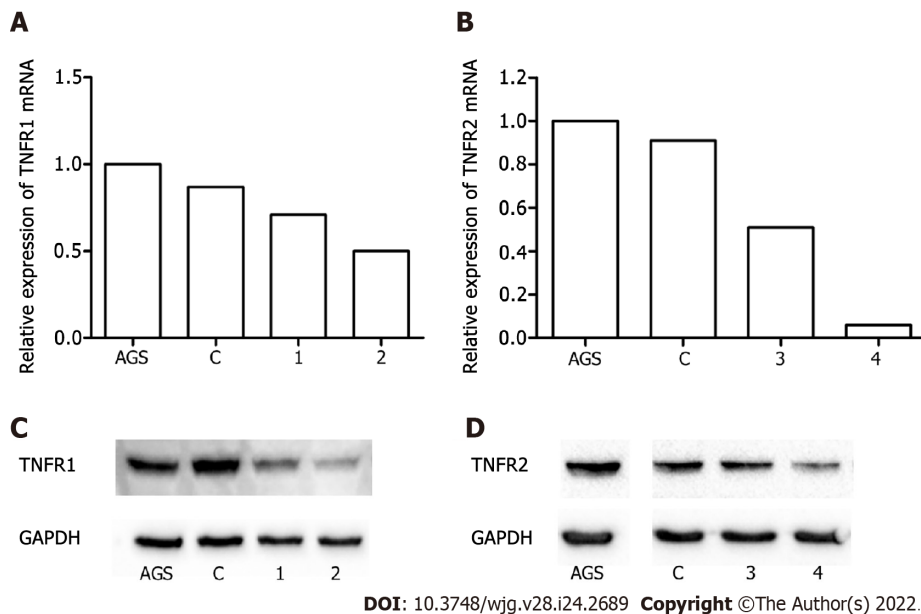


Figure 1 The efficiency of *TNFR1* and *TNFR2* expression silencing in AGS cells. A and B: Expression levels of *TNFR1* and *TNFR2* mRNA evaluated by real-time reverse transcription-quantitative polymerase chain reaction; C and D: Protein expression of *TNFR1* and *TNFR2* evaluated by Western blotting. Downregulation of *TNFR1* (A-C) and *TNFR2* (B-D) was confirmed by the reduction in the expression of receptors *TNFR1* and *TNFR2* following treatment with shRNAs 2 and 4, respectively. AGS: Nonsilenced cell line; C: Control transfected with empty vector; 1 and 2: Different shRNA clones for *TNFR1*; 3 and 4: Different shRNA clones for *TNFR2*.

treatment contributed to the differences observed in *NFKB1* and *NFKB2* mRNA expression ($P < 0.001$ for both).

***TNFR2* downregulation upregulates *miR-19a* and *miR-34a* expression**

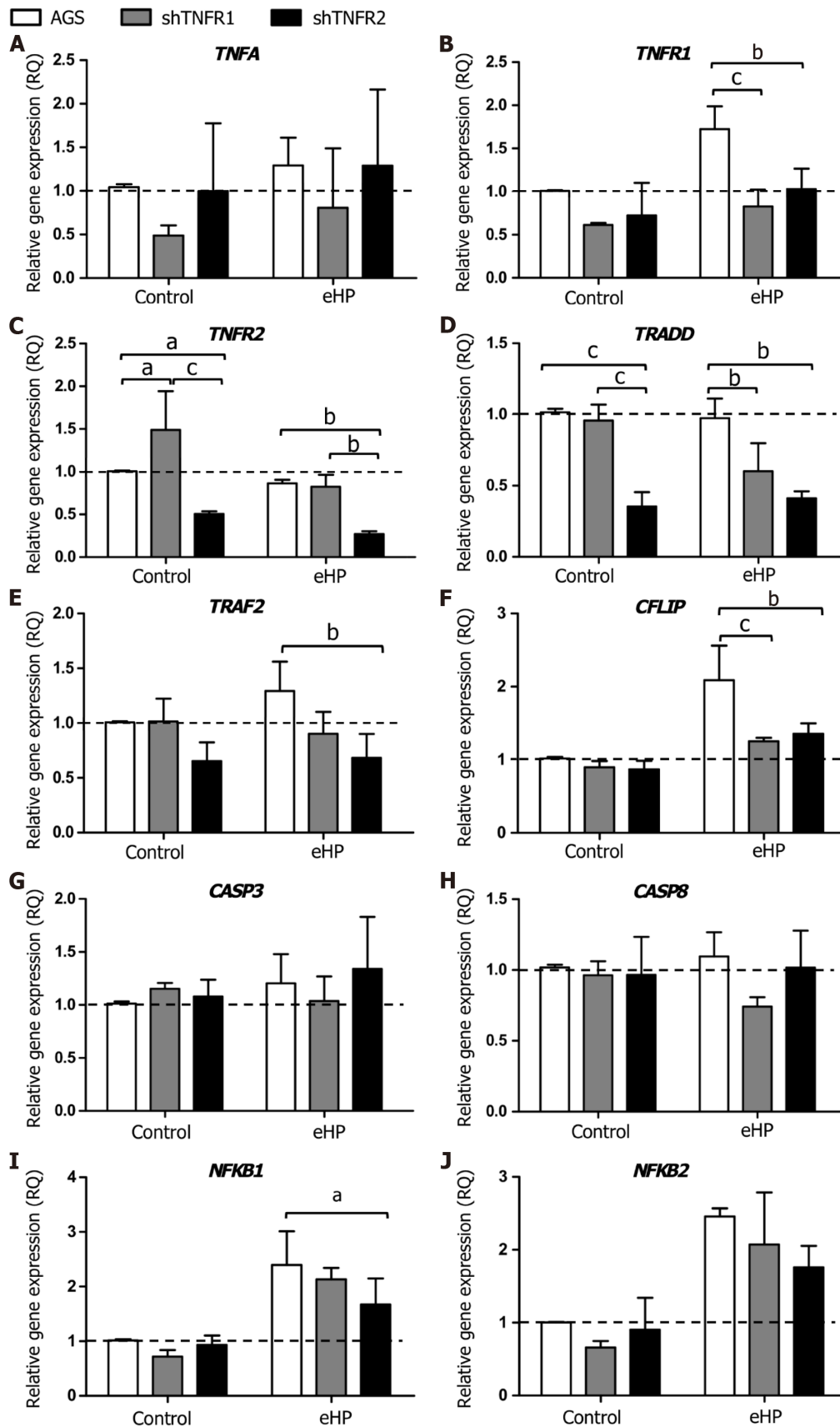
The expression of miRNAs *miR-19a*, *miR-34a*, *miR-103a*, *miR-130a* and *miR-181c* was evaluated in the AGS, sh*TNFR1* and sh*TNFR2* cell lines with and without *H. pylori* extract treatment (Figure 3 and Supplementary Figure 5). No significant change in the expression of these miRNAs was found in AGS and sh*TNFR1* cells regardless of *H. pylori* extract treatment. However, *TNFR2* receptor downregulation resulted in a significant increase in the expression of *miR-19a* and *miR-34a* in sh*TNFR2*-C cells (RQ = 3.70 and 2.64, respectively) compared with that of the AGS-C ($P < 0.01$ and $P < 0.001$, respectively) and sh*TNFR1*-C cells ($P < 0.05$ and $P < 0.001$, respectively) cells and in sh*TNFR2*-*H. pylori* extract cells (RQ = 3.89 and 1.69, respectively) compared to AGS-*H. pylori* extract ($P < 0.01$ and $P < 0.05$, respectively) and sh*TNFR1*-*H. pylori* extract cells ($P < 0.01$ and $P < 0.01$, respectively) (Figure 3A and B).

***TNFR2* downregulation promotes a reduction in G0/G1 phases and an increase in S phase**

The effect of *TNFR1* and *TNFR2* downregulation after *H. pylori* extract treatment on cell cycle progression was evaluated by flow cytometry. Overall, *H. pylori* extract treatment did not affect the cellular distribution in the G0/G1, S and G2/M phases in nonsilenced AGS, sh*TNFR1* and sh*TNFR2* cells (Supplementary Figure 6). However, *TNFR2* downregulation significantly reduced the number of cells in G0/G1 phase regardless of *H. pylori* extract treatment and led to an increase in the number of cells in S and G2/M phases in sh*TNFR2*-C cells compared to that of the AGS-C cells (Figure 4).

Downregulation of *TNFR2* promotes early apoptosis

In addition, we also investigated the effect of partial *TNFR1* and *TNFR2* inhibition and *H. pylori* extract treatment on apoptosis, which was evaluated through Annexin-V and PI double staining by flow cytometry. Treatment with *H. pylori* extract did not induce changes in the rates of early and late apoptosis in the nonsilenced AGS and both silenced AGS cell lines compared to the respective untreated cell lines (Supplementary Figure 7). However, after *H. pylori* extract treatment, the percentage of early apoptotic cells was significantly higher in sh*TNFR2* cells (1.27%) than in AGS (0.56%) and sh*TNFR1* cells (0.52%), whereas the percentage of late apoptotic cells was significantly reduced after *H. pylori* extract treatment (Figure 5). Together with the cell cycle results, these data indicate a relationship between decreased cell quantity in the G0/G1 phase and increased early apoptosis as a result of *TNFR2* downregulation.



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Figure 2 Relative expression of tumour necrosis factor- α signalling pathway genes. Expression levels of *TNFA*, *TNFR1*, *TNFR2*, *TRADD*, *TRAF2*, *CFLIP*, *CASP3*, *CASP8*, *NFKB1* and *NFKB2* mRNA in nonsilenced AGS, AGS with *TNFR1* downregulation (sh*TNFR1*), and AGS with *TNFR2* downregulation (sh*TNFR2*) either not treated (control) or treated with a *Helicobacter pylori* extract. A: *TNFA*; B: *TNFR1*; C: *TNFR2*; D: *TRADD*; E: *TRAF2*; F: *CFLIP*; G: *CASP3*; H: *CASP8*; I: *NFKB1*; J: *NFKB2*. Bars represent the mean \pm SD from three independent trials. The dotted line indicates relative quantification: RQ = 1.0. Statistically

significant difference: ^a $P < 0.05$; ^b $P < 0.01$; ^c $P < 0.001$. eHP: *Helicobacter pylori* extract.

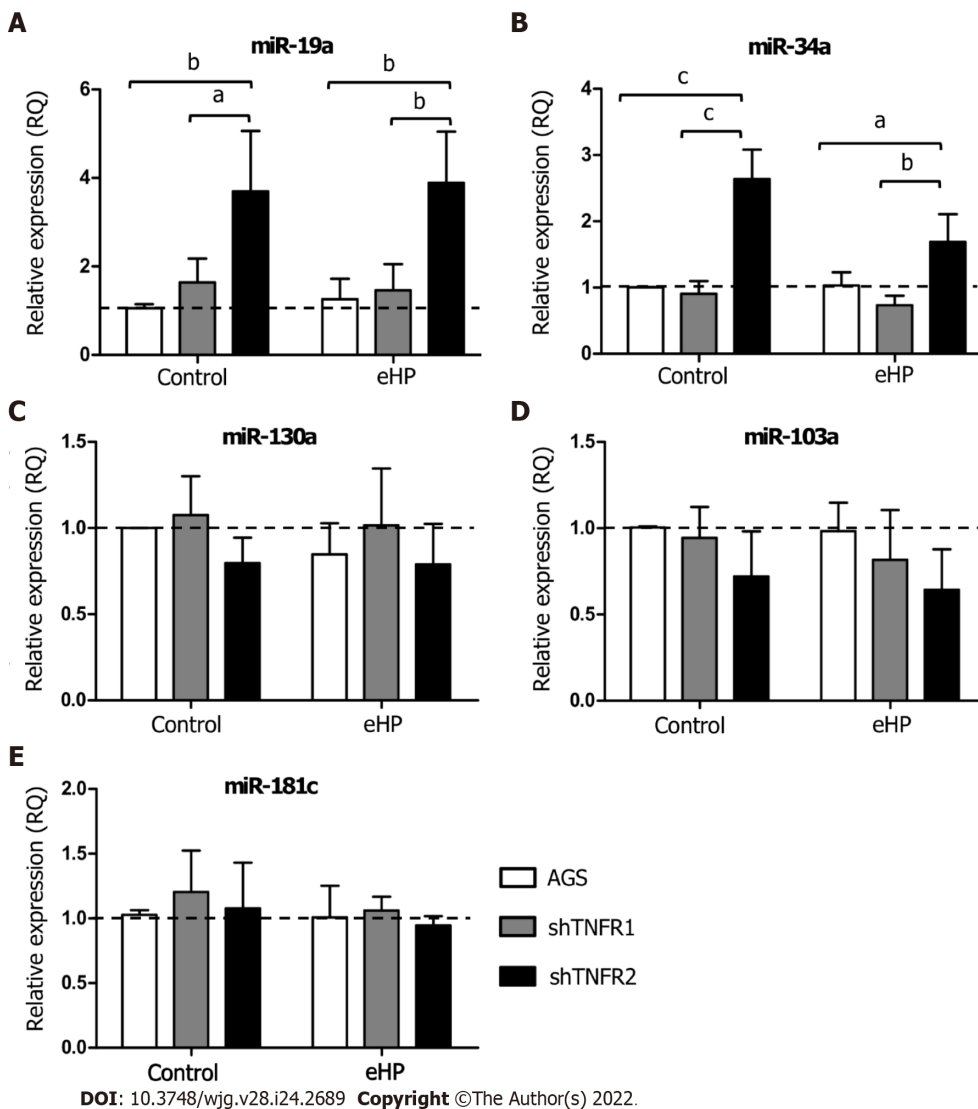


Figure 3 Relative expression of miRNAs related to the tumour necrosis factor- α pathway. Expression levels of miR-19a, miR-34a, miR-103a, miR-130a and miR-181c in nonsilenced AGS, AGS with TNFR1 downregulation (shTNFR1), and AGS with TNFR2 downregulation (shTNFR2) either not treated (control) or treated with the *Helicobacter pylori* extract. A: miR-19a; B: miR-34a; C: miR-103a; D: miR-130a; E: miR-181c. Bars represent the mean \pm SD from three independent trials. The dotted line indicates relative quantification: RQ = 1.0. Statistically significant difference: ^a $P < 0.05$; ^b $P < 0.01$; ^c $P < 0.001$. eHP: *Helicobacter pylori* extract.

DISCUSSION

TNFR1 and TNFR2 differ in their intracellular domains and cellular expression; thus, the role of each receptor in the TNF- α -triggered signalling pathway varies according to the pathological condition, activating different cellular processes, such as cell survival and apoptosis[10]. This is the first study to investigate the effect of TNFR1 and TNFR2 downregulation on AGS cells treated with *H. pylori* extract. Our results show that *H. pylori* extract treatment of nonsilenced AGS cells upregulated the mRNA expression of proliferation (*NFKB1* and *NFKB2*) and antiapoptotic genes (*CFLIP*) by activating TNFR1. TNFR1 downregulation did not promote extensive changes in the expression of genes or miRNAs involved in the TNF- α signalling pathway or in cellular processes. In contrast, TNFR2 downregulation significantly decreased *TRADD* and *TRAF2* mRNA expression, which may impair TNFR1-mediated TNF- α signalling, and increased miRNA expression, which promoted a block in the G₁/S transition and an increase in early apoptosis.

H. pylori infection can deregulate the expression of several genes and miRNAs, as previously shown by our research group[6]. Recently, increased mRNA levels of *TNFA* and other proinflammatory mediators, such as interleukin (IL)-1 β and IL-8, have been reported in an AGS cell line after infection by

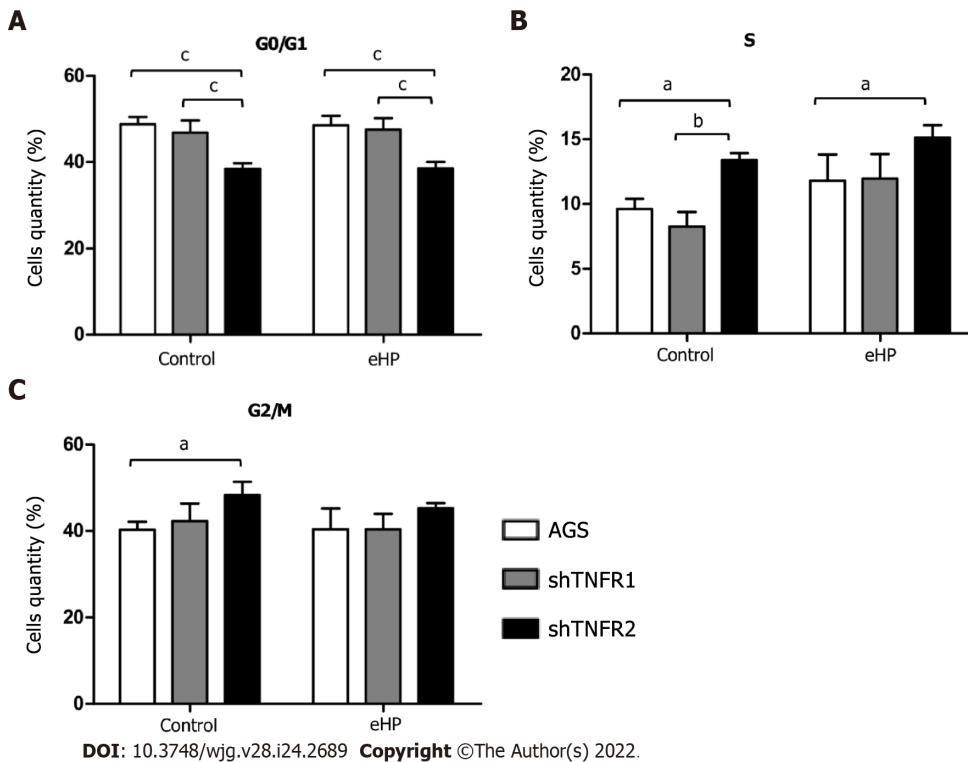


Figure 4 Cell cycle distribution analysis. Number of cells in the G0/G1, S and G2/M phases of the cell cycle in nonsilenced AGS cells, cells with *TNFR1* downregulated (sh*TNFR1*), and cells with *TNFR2* downregulated (sh*TNFR2*) without additional treatment (control) and those treated with the *Helicobacter pylori* extract. A: G0/G1; B: S; C: G2/M. Bars represent the mean \pm SD from three independent trials. Statistically significant difference: ^a $P < 0.05$; ^b $P < 0.01$; ^c $P < 0.001$. eHP: *Helicobacter pylori* extract.

H. pylori[19]. Our results show high *TNFR1* mRNA expression after *H. pylori* extract treatment in nonsilenced AGS cells, suggesting that *H. pylori* infection promotes activation of the *TNFR1*-mediated *TNF- α* signalling pathway in AGS cells, directing signal transduction to the cell survival pathway due to the increased expression of *NFKB1*, *NFKB2* and *CFLIP* (Figure 6A). In agreement with this finding, previous studies showed that *H. pylori* activates NF- κ B in a manner dependent on the bacterial cell number[20] due to significant I κ B- α degradation[21] resulting in the induction of anti-apoptotic gene transcription, such as *CFLIP* transcription[22]. Therefore, our results indicate that *H. pylori* extract also induces cell survival and inflammation due to *TNFR1*-mediated *TNF- α* signalling, which leads to NF- κ B activation and the consequent production of antiapoptotic mediators, such as *cFLIP*.

TNFR1 downregulation resulted in a significant increase in *TNFR2* mRNA expression in nontreated AGS cells, while a decrease in the expression of *TRADD* and *CFLIP* mRNA was observed in cells treated with *H. pylori* extract, although no change was observed in apoptosis and cell cycle assays (Figure 6B). In oesophageal carcinoma cells treated with *TNFR1*-siRNA, Changhui *et al*[23] demonstrated an increase in cell proliferation and a reduction in apoptosis rate after 24 h of transfection, and the cell proliferation level was time-dependent[19]. The present study, when evaluated together with the results of *H. pylori* extract, suggests that the assays of cellular processes after 6 h of treatment may have been insufficient in sh*TNFR1* and nonsilenced AGS cells. Wan *et al*[24] showed that AGS cell coculture with *H. pylori* inhibited apoptosis and increased viability through upregulation of *TRAF1*, which was triggered by NF- κ B activation. However, the peak of *TRAF1* expression occurred after 12 h of infection, and its effect on cell viability started only after 24 h. Therefore, the role of *TNFR1* downregulation in GC cell lines still needs to be further investigated.

Conversely, the downregulation of *TNFR2* expression significantly decreased the expression of two important mediators of the *TNFR1*-mediated signalling pathway, *TRAF2* and *TRADD*, in addition to *NFKB1*, *CFLIP* and *TNFR1* expression. Moreover, there was an increase in early apoptosis, with concomitant G1/S transition phase arrest (Figure 6C). These results agree with those previously reported by our group in GC patient samples, in which we found upregulation of *TNF/TNFR2* and cellular survival genes such as *TRAF2*, *CFLIP*, and *NFKB2* and downregulation of *TNFR1* and *CASP3* [14], thus emphasizing the important role of *TNFR2* in gastric carcinogenesis and *TNFR2* silencing as a promising strategy for anticancer therapy[25].

TRADD is an essential adaptor protein that functions in *TNFR1*-mediated apoptotic signalling under physiological conditions[26]. However, the dependence of this mediator seems to be cell-type specific [27], as *TRADD* knockout mice were resistant to *TNFR1*-induced toxicity in a hepatitis model[28]. However, this pathway was not completely impaired in macrophages deprived of *TRADD*[27].

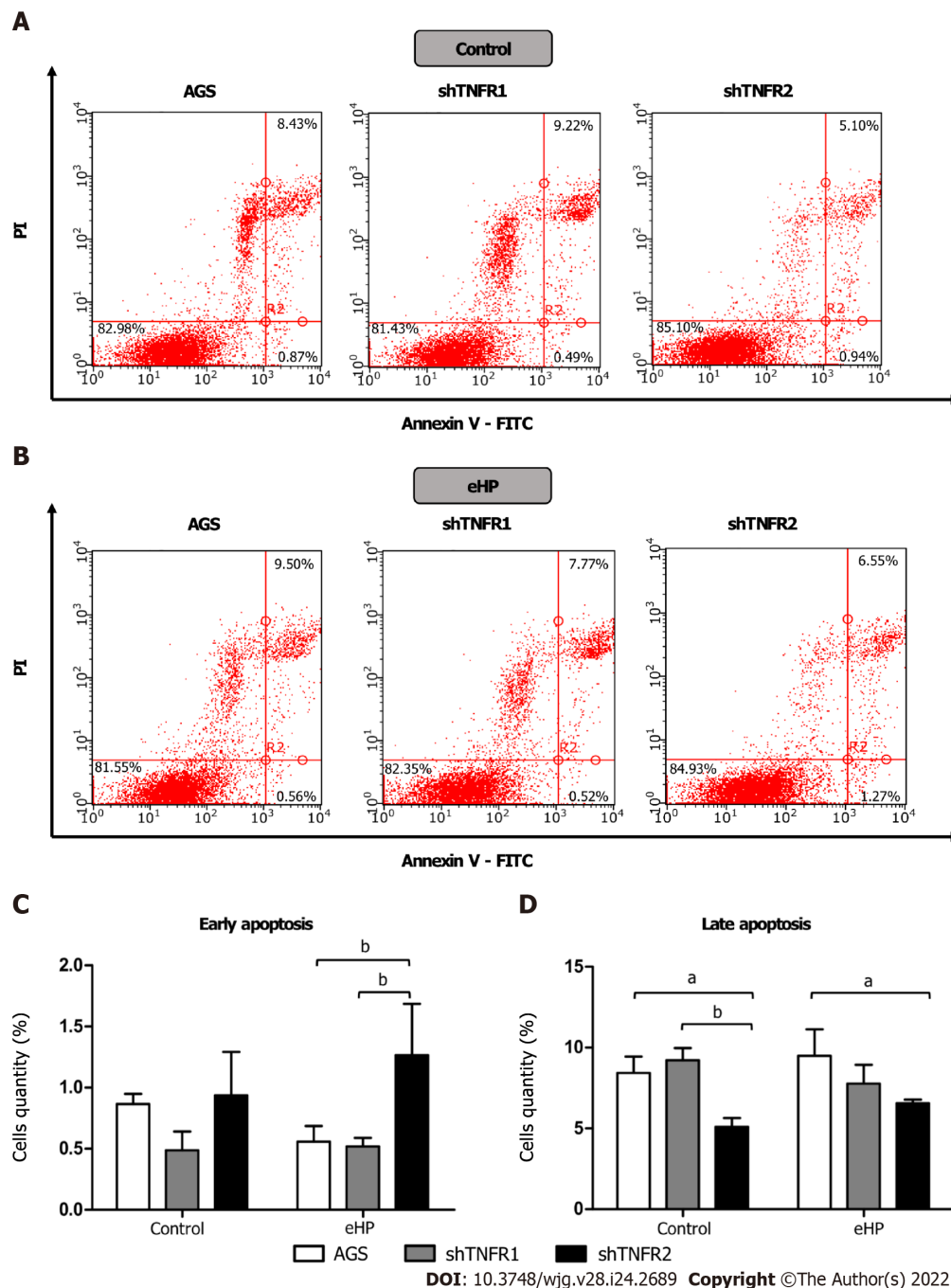
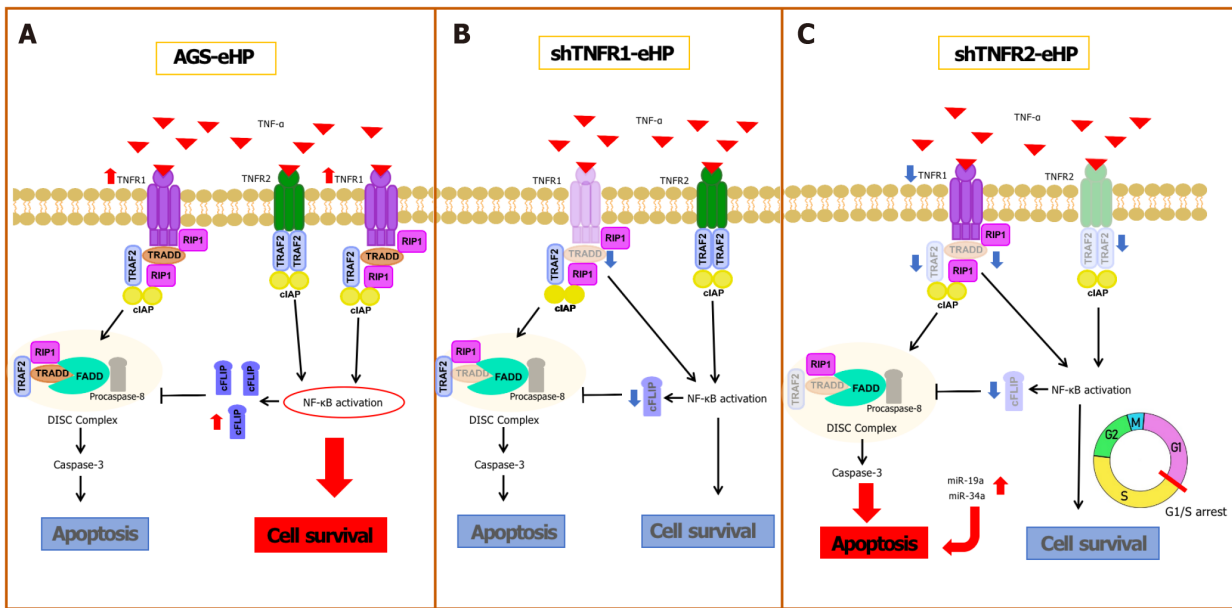


Figure 5 Apoptosis distribution analysis in nonsilenced AGS cells, cells with TNFR1 downregulated (shTNFR1), and cells with TNFR2 downregulated (shTNFR2). A: Cells without additional treatment (control); B: Cells treated with the *Helicobacter pylori* extract; A and B: Representative images of the cell distribution and percentage in the different quadrants indicating viable cells (Annexin V-/PI-) in the lower left, early apoptotic cells (Annexin V+/PI-) in the lower right and late apoptotic cells (Annexin V+/PI+) in the upper right; C and D: Histogram bars represent the mean \pm SD from three independent trials for early (C) and late (D) apoptosis. Statistically significant difference: ^a $P < 0.05$; ^b $P < 0.01$. eHP: *Helicobacter pylori* extract.

Therefore, apoptosis can occur even with reduced expression of TRADD, as observed in the present study after TNFR2 downregulation. In turn, TRADD inhibition reduces NF- κ B activation[12], impairing the transcription of anti-apoptotic genes, such as *TRAF2* and *CFLIP*, as observed in this study.

As mentioned, shTNFR2 cells also exhibited reduced expression of TRAF2. This protein has an essential role in signal transduction and is triggered by both TNFR1 and TNFR2, suggesting the existence of crosstalk between them[29], which influences the signalling outcome after TNF binding [30]. TRAF2 recruits cIAP1 and cIAP2 after interaction with these receptors, thereby triggering nuclear translocation of NF- κ B[30]. Therefore, TRAF2 is a negative regulator of TNF-induced apoptosis[30], and the use of this protein after TNFR2 activation results in TNFR1-induced apoptosis to the detriment of proliferation[25]. Therefore, a decrease in TRAF2 expression favours TNFR1-mediated apoptosis, contributing to the increase in the percentage of early apoptotic cells after TNFR2 downregulation.



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Figure 6 Schematic comparison of the tumour necrosis factor- α signalling pathway and cell fate in nonsilenced AGS, shTNFR1 and shTNFR2 cells with downregulation after treatment with *Helicobacter pylori* extract. After the tumour necrosis factor (TNF)- α /TNFR1 interaction, TRADD, RIP1, TRAF2 and cellular inhibitor of apoptosis proteins are recruited, which results in nuclear factor-kappaB (NF- κ B) activation and transcription of prosurvival and antiapoptotic genes, such as CFLIP, that inhibit the activity of the death-inducing signalling complex, which is formed after dissociation between TRADD and TNFR1. As with TNFR1, TNFR2 stimulation leads to NF- κ B activation and cell survival; A: Treatment with *Helicobacter pylori* (*H. pylori*) extract resulted in increased expression of TNFR1, NFKB1, NFKB2 and CFLIP mRNAs in nonsilenced AGS cells. Therefore, the cell survival pathway is activated due to NF- κ B activation (red ellipse) through the TNFR1-TNF signalling pathway with consequent production of the antiapoptotic CFLIP; B: In shTNFR1-*H. pylori* extract cells, TNFR1 downregulation resulted in decreased expression of TRADD and CFLIP mRNAs; however, it did not change cell fate; C: In shTNFR2-*H. pylori* extract cells, TNFR2 downregulation decreased the expression of TRAF2 and TRADD, decreased NFKB1, CFLIP and TNFR1 expression, and upregulated miR-19a and miR-34a. This led to an increase in apoptosis, impairing cell survival due to arrest in the G1/S transition phase.

Moreover, we found that TNFR2 downregulation also promoted a decrease in the ratio of cells in the G0/G1 phase and an increase the ratio of cells in the S and G2/M phases, suggesting that TNFR2 inhibition may delay cell cycle progression and arrest cells at the G1/S transition[31]. These changes in the cell cycle could also be related to the DNA damage response[32], leading cells to early apoptosis, as seen in our results. Recent studies have shown increased expression of genes related to the response to DNA damage and repair, such as *APE1*, *H2AX* and *PARP-1*, in GC samples, thus possibly influencing the survival of tumour cells[33,34]. Yang *et al*[35] demonstrated that TNFR2 enhances DNA damage repair by regulating PARP expression in breast cancer cells. Furthermore, they showed that TNFR2 silencing led to an increase in pH2AX, which is a DNA damage marker.

Based on the function of each receptor and the existence of crosstalk between them, it is possible that the simultaneous inhibition of both TNFR1 and TNFR2 may have a greater antitumor effect on GC than the downregulation of TNFR2 alone. Furthermore, TNF- α and its receptors, in addition to being expressed by tumour cells, are also expressed by cells present in the tumour microenvironment as immune cells[36]. Oshima *et al*[8] demonstrated that the stimulation of TNFR1-mediated TNF- α signalling in cells in the tumour microenvironment promoted gastric tumorigenesis through the induction of tumour-promoting factors (*Nox1* and *Gna14*) in tumour epithelial cells, highlighting the importance of the tumour microenvironment[37] and the need for *in vivo* studies.

Furthermore, considering the important role of miRNAs in the development of cancer, as they act as regulators of signalling pathways and are consequently involved in various cellular processes, we also evaluated the expression of miRNAs miR-19a, miR-34a, miR-103a, miR-130a and miR-181c. These miRNAs were chosen from data analysis published in public databases[38-42] and due to their interaction with TNF- α pathway genes, as observed in our recent study[14]. Regardless of *H. pylori* extract treatment, shTNFR1 and nonsilenced AGS cells did not dysregulate miRNA expression. However, TNFR2 downregulation caused an upregulation of miR-19a and miR-34a (Figure 3A and B).

MiR-34a is dysregulated in different types of cancer and is linked to the proliferation, differentiation, migration, invasion, treatment, and prognosis of cancer[43]. Although most studies suggest a tumour-suppressor role for miR-34a[44-46], we recently observed overexpression of this miRNA in GC samples [14], thus also indicating its oncogenic action. In contrast, in both AGS and BGC-823 cell lines, an upregulation of this miRNA inhibited proliferative and migratory abilities in sevoflurane-induced GC cells, whereas *in vivo* knockdown of miR-34a stimulated tumour growth, indicating its action as a tumour suppressor in the AGS cell line[47]. In addition, stable transfection of pre-mir-34a in KatoII cells

increased the percentage of apoptotic cells and reduced the proliferation rate, suggesting that its high expression promotes apoptosis[48]. In the present study, upregulation of this miRNA in shTNFR2 cells suggests that miR-34a expression can be modulated by TNFR2-mediated TNF- α signalling and is able to exert an anti-proliferative and pro-apoptotic effect. Similarly, miR-19a was overexpressed in the shTNFR2 cell line, indicating that miR-19a and TNFR2 are related to each other in GC. miR-19a has a known oncogenic function in various kinds of cancer[49], with *TNFA*, *TNFR1* and *TNFR2* being its validated targets[38,39,50]. However, miR19a may play a tumour-suppressive role, as reported in prostate cancer, by suppressing invasion and migration in bone metastasis[51], and in rectal cancer cells, by inducing apoptosis[52]. For both miRNAs, functional studies are needed to assess what targets the miRNAs are related to with respect to TNF- α signalling in GC.

The other miRNAs evaluated (miR-103a, miR-130a and miR-181c) were not dysregulated in the AGS cell line. Although different studies have shown the involvement of these miRNAs in gastric neoplasms [53-55], their pro- or antitumor roles are still controversial. This is due to the influence of several factors in the regulation of gene expression, such as the stage of tumour development and the presence or absence of infectious agents[6].

CONCLUSION

In conclusion, based on our results, shRNA-mediated downregulation of TNFR2 in AGS cells was able to reduce the expression of pro-survival and anti-apoptotic genes, in addition to affecting miRNA expression, the cell cycle, and promoter apoptosis. The blocking of TNFR2 expression may cause antitumor effects, suggesting possible targets for future studies into therapeutic strategies for treating GC. Furthermore, the *H. pylori* extract increased the expression of prosurvival genes, mainly through TNFR1-mediated TNF- α signalling, thus emphasizing the role of bacterial infection in promoting GC progression.

ARTICLE HIGHLIGHTS

Research background

The tumour necrosis factor- α (TNF- α) signalling pathway triggered by TNFR1 and TNFR2 controls several biological processes, influencing cell fate. Thus, the deregulation of this pathway to cause an imbalance between the processes of cell survival and death may contribute to the tumorigenic process. This variety of functions is exercised by the ability of TNF- α to bind to TNFR1 or TNFR2, which results in different cellular processes. Both receptors are transmembrane proteins and are largely similar in extracellular structure, but their intracellular domains are different, and dictate the cellular fate for either survival or death. Since only TNFR1 has a death domain, the TNF- α signalling pathway triggered by TNFR1 is able to induce both cell survival and apoptosis, whereas TNFR2 results only in cell survival. The TNF- α signalling pathway also modulates the immune response and inflammation, so deregulation of this pathway has been implicated in inflammatory diseases and cancer. Therefore, studies are needed to better understand the relationships of this signalling network *via* TNFR1 and TNFR2 and its protumorigenic or antitumorigenic effects.

Research motivation

We proposed the present study based on our previous studies, which showed deregulation in the expression of genes and miRNAs of the TNF- α signalling pathway and its receptors TNFR1 and TNFR2 in fresh tissues of chronic gastritis and gastric cancer (GC) patients. Therefore, we decided to evaluate the effect of silencing TNFR1 and TNFR2 on GC cell lines.

Research objectives

According to the role of TNFR1 and TNFR2 in cellular responses triggered by TNF- α , and considering that studies addressing the role of these receptors in gastric neoplasm are limited and inconclusive, we proposed to couple the silencing of TNFR1 and TNFR2 receptors in an AGS gastric cell line and the treatment with *Helicobacter pylori* (*H. pylori*) extract to determine the effects on TNF- α mRNA expression and on downstream genes related to its signalling pathway. Moreover, we also investigated previously studied miRNAs that target genes in the TNF- α pathway to jointly determine their influence on the cell cycle and apoptosis.

Research methods

Stable AGS GC cells containing TNFR1 and TNFR2 receptors downregulated by specific shRNAs and nonsilenced cell lines were treated with 30% v/v *H. pylori* extract [*H. pylori* Tox+ strain (cagA+/vacA s1m1)] for 6 h. After silencing, TNFR1 and TNFR2 levels were assessed by quantitative polymerase

chain reaction (qPCR) and Western blotting to confirm the knockdown effect. Subsequently, mRNA and miRNAs were quantified by qPCR using TaqMan gene and miRNA expression assays. The MTT assay was employed to evaluate the viability of silenced and nonsilenced AGS cells after different treatment conditions with *H. pylori* extract, and flow cytometry was used for cell cycle analysis and apoptosis.

Research results

Our results showed that *H. pylori* extract treatment increased the expression of genes involved in cell survival (*NFKB1* and *NFKB2*) and inhibited apoptosis (*CFLIP*) and *TNFR1* in nonsilenced AGS cells. *TNFR1* downregulation significantly decreased the expression of the *TRADD* and *CFLIP* genes; however, no change in the cell cycle, apoptosis or miRNA levels was observed. In turn, *TNFR2* downregulation decreased the expression of the *TRADD* and *TRAF2* genes, which are both important downstream mediators of the *TNFR1*-mediated pathway, as well as the *NFKB1* and *CFLIP* genes, while upregulating the expression of miR-19a and miR-34a. Consequently, there was a decrease in the ratio of cells in the G0/G1 phase and an increase in cells in the S phase, as well as the promotion of early apoptosis.

Research conclusions

Our findings highlight that treatment with *H. pylori* extract increased the expression of pro-survival genes, mainly through *TNFR1*-mediated TNF- α signalling, emphasizing the role of bacterial infection in promoting GC progression. In the AGS cell line, *TNFR1* and *TNFR2* downregulation decreased the expression of prosurvival and antiapoptotic genes and affected miRNA expression and cellular processes, such as the cell cycle and apoptosis, emphasizing that shRNA-mediated downregulation of these receptors can have an antitumor effect.

Research perspectives

According to our results, we can mainly highlight the important role of *TNFR2* in the TNF- α pathway in GC, indicating that silencing *TNFR2* can reduce the expression of survival and anti-apoptotic genes. Thus, blocking this receptor may result in antitumor effects, suggesting possible targets for future *in vivo* studies into therapeutic strategies for treating GC.

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FOOTNOTES

Author contributions: Rossi AFT analysed and interpreted the results; Rossi AFT, Manoel-Caetano FS and Biselli JM performed the experiments; Rossi AFT and Silva AE outlined the study, and drafted and revised the manuscript; Cabral AS performed the Western blot assays; Saiki MFC provided technical support for the shRNA transfection experiments; Ribeiro ML provided the HGC-27 cell line and produced the *H. pylori* extract; all authors approved the final version of the manuscript.

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