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Inflammatory mediators, tumor necrosis factor-α and interferon-γ, induce EMT in human PTC cell lines

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Abstract. Inflammatory mediators, tumor necrosis factor (TNF)-α and interferon (IFN)-γ, promote adverse outcomes in numerous types of cancer; however, their role in papillary thyroid cancer (PTC) remains unclear. The aim of the present study was to investigate the influence of TNF-α and IFN-γ on the migration, invasion and epithelial-mesenchymal transition (EMT) of the three PTC cell lines, TPC-1, BCPAP and K1. The effect of TNF-α and IFN-γ on cell migration and invasion was assessed by wound-healing and Transwell assays. In addition, the mRNA and protein expression levels of the EMT makers, E-cadherin, N-cadherin and vimentin, were analyzed using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and immunoblot analysis. The wound-healing and Transwell experiments revealed that TNF-α and IFN-γ increased the migratory and invasive behavior of PTC cells ($P<0.05$). RT-qPCR revealed that TNF-α and IFN-γ downregulated E-cadherin mRNA, while they upregulated N-cadherin and vimentin mRNA expression levels. These results were further confirmed by the immunoblot analysis. The results of the present study suggest that TNF-α and IFN-γ induce EMT and malignant progression in human PTC cells.

Introduction

Thyroid cancer is the most common endocrine carcinoma, with papillary thyroid carcinoma (PTC) accounting for ~80% of all thyroid cancer cases (1). PTC is well-differentiated and prone to metastasizing to the regional lymph nodes. The incidence and mortality of PTC are increasing rapidly worldwide, as well as in China (2,3). Reports have demonstrated the incidence and mortality rates of thyroid cancer were raised at a rate of 14.51% and 1.42% respectively, each year between 2003-2007 in China (4). The majority of cancer-associated mortalities are caused by metastasis, a process that involves changes in the cancer cells, but also in the tumor microenvironment. Tumor cells and/or tumor-associated leukocytes and platelets can produce inflammatory cytokines that may contribute directly towards malignant progression (5). A previous study demonstrated that human thyroid samples express genes coding the majority of inflammatory proteins that were identified in cell cultures, including CXCR4, CD44, OPN, CXCL1, CXCL10 and SDF-1 (6). The oncogenes activated in thyroid carcinomas, including *RET/PTC*, *RAS* and *BRAF*, which trigger the MAPK cascade, can induce a cell-autonomous proinflammatory transcriptional program in thyrocytes, which includes expression of cytokines, chemokines and their receptors (7). Proinflammatory cytokines, such as tumor necrosis factor (TNF)-α and interferon (IFN)-γ, are important inflammatory mediators of these processes (8,9). TNF-α and IFN-γ are pleiotropic cytokines expressed in various types of tumor cells and cells in the tumor microenvironment, exerting a variety of pro-tumoral activities in solid tumors (10,11). Recent research into the inflammatory microenvironment of malignant PTC tissues has supported the hypothesis that a close association exists between inflammation and tumor metastasis progression (12,13). Despite attempts in the last few decades to elucidate the inflammatory cytokines underlying invasion and metastasis in PTC patients, the understanding about the role of TNF-α and IFN-γ in PTC is limited.

Epithelial-mesenchymal transition (EMT) is a complex process during which epithelial cells lose intercellular adhesion, acquire fibroblast-like characteristics and increase migratory and invasive properties. EMT was initially reported in embryonic development; however, it also occurs during tumor metastasis (14,15) and appears to be common in PTC invasion (16-19). Transforming growth factor-β (TGF-β) and epidermal growth factor (EGF) are inflammatory cytokines that are able to stimulate EMT in thyroid cancer cells or thyroid cells cultured *ex vivo* (20,21). Furthermore, chronic low levels

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of TNF-α and IFN-γ have been demonstrated to induce invasion and metastasis of cancer (11,12,22-24) via mechanisms involving the SMAD, NF-κB, AKT/GSK-3β and JAK/STAT signaling pathways. Thus, EMT represents a convergence point between inflammation and the progression of cancer (25); however, the mechanisms through which inflammation is involved in the different stages of tumor invasion, intravasation and subsequent metastasis to the distant organ sites remain poorly defined (26).

In the present study, the effects of TNF-α and IFN-γ on the migration and invasion of various PTC cell lines were investigated. In addition, the association of TNF-α and IFN-γ with the expression levels of E-cadherin, N-cadherin and vimentin was examined. The current study aimed to provide a basis for the investigation of the chronic inflammatory microenvironment and EMT in PTC tissues.

Materials and methods

Cell culture. The PTC cell line, BCPAP (harboring the *BRAF* mutation), was purchased from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH (Braunschweig, Germany). In addition, the PTC cell line, K1 (harboring the *BRAF* mutation), was purchased from the Health Protection Agency Culture Collections (Salisbury, UK). BCPAP and K1 cells were cultured in RPMI 1640 medium. The PTC cell line, TPC-1 (harboring the *RET/PTC* mutation), was acquired from Dr Bryan R. Haugen of the Division of Endocrinology, Diabetes and Metabolism, University of Colorado Denver (Aurora, CO, USA) and cultured in high-glucose Dulbecco's modified Eagle's medium. All culture media were supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) and cells were cultured in a humidified atmosphere containing 5% CO_2 at 37˚C. All culture reagents were purchased from Life Technologies (Grand Island, NY, USA).

Wound-healing assay. Cells ($2x10^5$/ml) were seeded in a 12-well plate at 80% cell confluence, and stimulated with 20 ng/ml TNF-α (Invitrogen Life Technologies, Grand Island, NY, USA) and 50 U/ml IFN-γ (Roche Applied Sciences, New York, NY, USA) for 12 h, and then the culture medium was replaced with fresh medium. Cells treated only with medium were regarded as control groups. After 24 h, a scratch wound in the monolayer was created using a sterile 10 µl pipette tip. Phase contrast images were captured between 0 and 24 h using a DMi1 inverted microscope (Leica Microsystems, Wetzlar, Germany). Data are presented as the percentages of the remaining gap distance relative to the initial gap distance, and are expressed as the mean ± standard deviation (SD) measurements from three independent experiments.

Transwell-invasion assay. Costar Transwell® chambers (pore size, 8 µm; Corning, Inc., Corning, NY, USA) were coated with 200 µl Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) at a 1:7 dilution and incubated overnight. The cells were co-cultured with 20 mg/ml TNF-α or 50 U/ml IFN-γ for 12 h, followed by incubation for 24 h in fresh culture medium. Next, the cells were seeded in the top chamber and medium containing 10% FBS was added to the lower chamber as a chemoattractant. After 24 h, the cells were fixed in 4% formaldehyde and stained with hematoxylin and eosin (Beyotime Institute of Biology, Suzhou, China). Cells that invaded through the pores to the lower surface of the filter were counted under a microscope (DMi1; Leica Microsystems). Data are expressed as the mean ± SD of triplicate measurements from three independent experiments.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA extraction, cDNA synthesis, and qPCR were performed as previously described (27). Briefly, total RNA was extracted from the cells using TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. RNA integrity was verified by 1.5% agarose gel electrophoresis, followed by staining with ethidium bromide (Sigma-Aldrich, St. Louis, MO, USA). The OD_{260}/OD_{280} absorbance ratio (where OD is the optical density at 260 and 280 nm, respectively) was between 1.9 and 2.0 in each RNA sample. Next, 1 mg total RNA was used to prepare cDNA. A reverse transcriptase kit (PrimeScript™ RT Reagent kit; Takara Biotechnology Co., Ltd., Dalian, China) was used for complementary DNA (cDNA) synthesis, at 37˚C for 15 min, followed by 85˚C for 5 sec, on an ABI 9700 GeneAmp® PCR system (Applied Biosystems Life Technologies, Grand Island, NY, USA). The transcripts were quantified using a Rotor-Gene 3000 Real-Time PCR system (Qiagen, Manchester, UK) and SYBR® Premix Ex *Taq*™ II (Takara Biotechnology Co., Ltd.), according to the manufacturer's instructions. Reactions began with a 10-sec hot-start activation of the *Taq* polymerase at 95˚C, followed by 40-45 cycles of amplification in three steps (denaturation at 95˚C for 5 sec, followed by 30-sec annealing at 60˚C and 30-sec extension at 72˚C). The primers used in each reaction were as follows (28): E-cadherin forward, 5'TGCCCAGAAAATGAAAAAGG-3, and reverse, 5'-GTGTATGTGGCAATGCGTTC-3'; N-cadherin forward, 5'-GAGAACTTTGCCGTTGAAGC-3', and reverse, 5'-GTGTATGTGGCAATGCGTTC3'; vimentin forward, 5'-GAGAACTTTGCCGTTGAAGC-3', and reverse, 5'-GCTTCCTGTAGGTGGCAATC-3'; GAPDH forward, 5'-ACCCAGAAGACTGTGGATGG-3', and reverse, 5'-TCTAGACGGCAGGTCAGGTC-3'. Data are expressed as the mean ± SD of three independent experiments.

Immunoblot analysis. Cells ($1x10^5$/ml) in 6-well plates were cultured until 60% confluence and then incubated with TNF-α (10 ng/ml, 20 ng/ml, 40 ng/ml) or IFN-γ (25 U/ml, 50 U/ml, 100 U/ml) for 36 h. Total cell extracts were prepared from the cell cultures using radioimmunoprecipitation assay buffer (Sigma-Aldrich) on ice. The extracts were centrifuged at 11,739 x g for 20 min, and the supernatant was subjected to a bicinchoninic acid assay for protein quantification. The samples were then boiled for 10 min at 100˚C. Proteins were resolved on a 10% gradient sodium dodecyl sulfate-polyacrylamide gel and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Subsequently, the membranes were blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline/Tween 20 (TBST) for 2 h at room temperature, and then

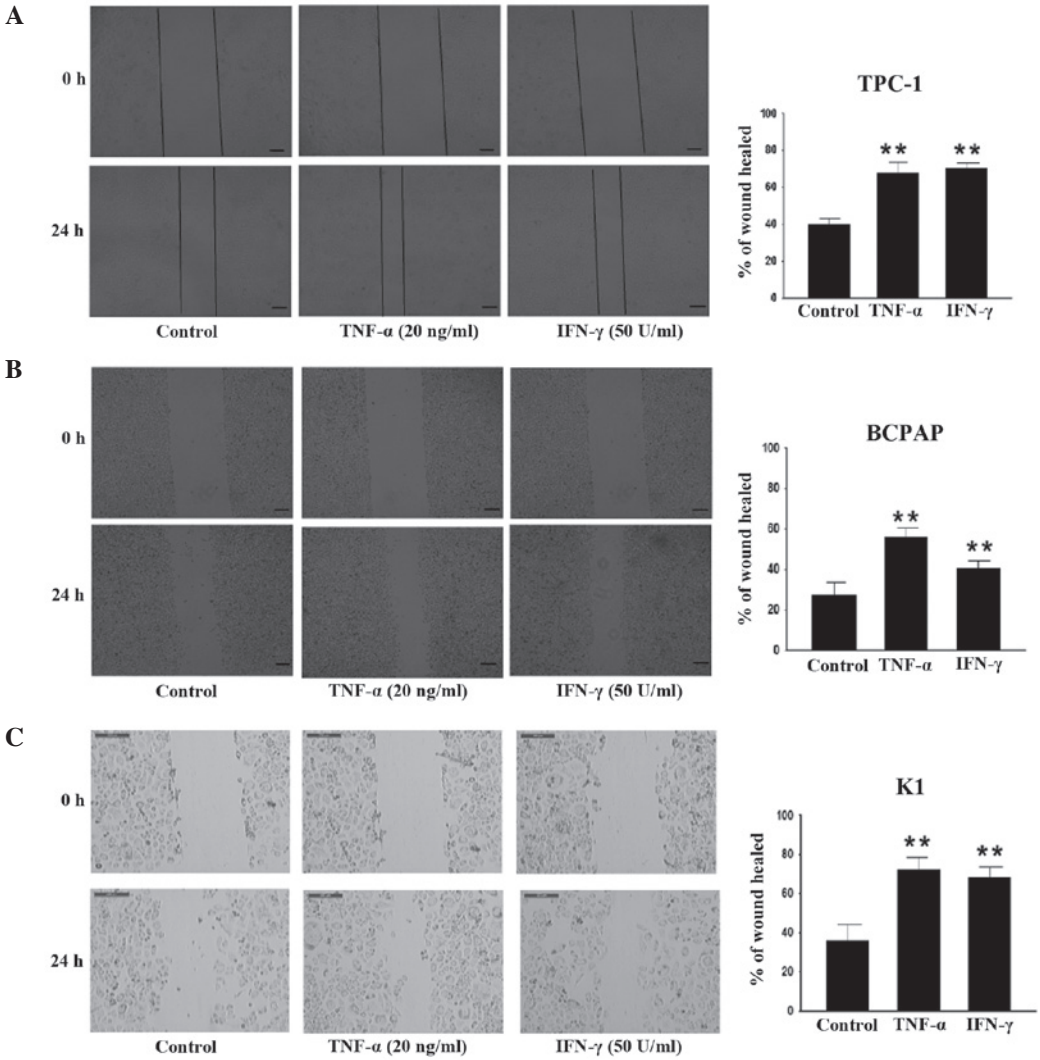

Figure 1. TNF-α and IFN-γ promote migration of PTC cells. For the wound-healing assay, the human PTC cell lines, (A) TPC-1, (B) BCPAP and (C) K1, were treated with 20 mg/ml TNF-α and 50 U/ml IFN-γ. Images were captures using a light microscope 24 h after scratching a wound in the monolayer (magnification, x40 for TPC-1 and BCPAP cells, x100 for K1 cells). The results from three independent experiments are presented as graphs of the mean ± standard deviation (*P<0.05 and **P<0.01, vs. control). Bars, 100 μm for TPC-1 and BCPAP cells, 200 μm for K1 cells. PTC, papillary thyroid carcinoma; TNF, tumor necrosis factor; IFN, interferon.

incubated with primary antibodies (dilution, 1:1,000 in 2.5% BSA/TBST). The following mouse monoclonal antibodies were used: Anti-E-cadherin (cat no. 610812, BD Biosciences); and anti-N-cadherin (cat no. sc-59987), anti-vimentin (cat no. sc-66002) and anti-β-actin (cat no. sc-47778) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Next, the samples were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (dilution, 1:5,000 in TBST; Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China). The densitometry of the immunoblot analysis results was measured using the ImageJ software (version 1.4w3u; National Institutes of Health, Bethesda, MD, USA) and data from three independent experiments.

Statistical analysis. Statistical analyses were performed using SPSS software (version 18.0; SPSS Inc., Chicago, IL, USA). The results are represented as the mean ± SD. Differences between the groups were determined using one-way ANOVA. P<0.05 was considered to indicate a statistically significant difference.

Results

TNF-α and IFN-γ promote migration and invasion of PTC cells. In order to explore whether TNF-α and IFN-γ can contribute to the metastasis and invasion of PTC cells *in vitro*, a wound-healing assay was used to assess cell migration following stimulation with TNF-α and IFN-γ. Compared with the control groups, incubation of the three thyroid carcinoma cell lines (TPC-1, BCPAP and K1) with TNF-α (20 ng/ml) significantly enhanced cell motility by ~2-fold (P<0.01; Fig. 1). The migration ability of the cells under the stimulation of INF-γ (50 U/ml) was similar to this, the motility of K1 and TPC-1 was increased by ~2-fold and the migration ability of BCPAP was increased by ~1.5-fold compared with the control (P<0.01; Fig. 1). Similarly, significant changes in cell invasive ability were observed compared with the control (P<0.01; Fig. 2). Following incubation with TNF-α and IFN-γ, the number of invasive TPC-1 cells was increased by ~6-fold, in comparison to cells incubated without any cytokines. In addition, subsequent to incubation of BCPAP cells with TNF-α, the fraction of invasive cells increased by

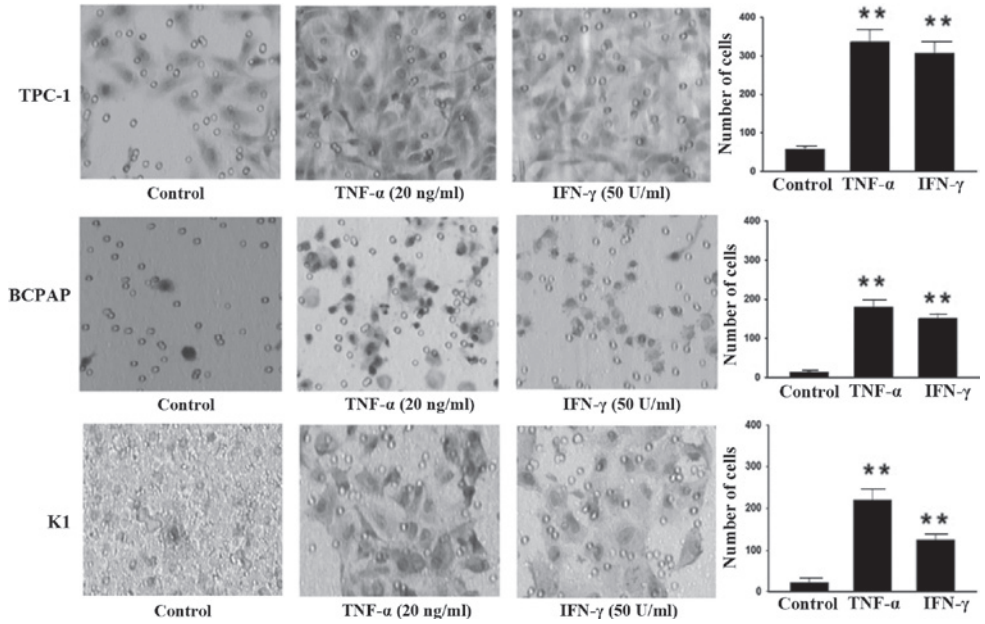

Figure 2. TNF-α and IFN-γ induce invasion in PTC cell lines. Cells were co-cultured with 20 mg/ml TNF-α and 50 U/ml IFN-γ, plated in the upper chamber of the Transwell and allowed to grow for 24 h in serum-free medium; then, 10% fetal bovine serum was placed in the lower chamber. The number of cells that invaded through the Matrigel was counted in 10 fields under a x40 objective lens. Error bars correspond to the mean ± standard deviation (**P<0.01 vs. control; magnification, x200). PTC, papillary thyroid carcinoma; TNF, tumor necrosis factor; IFN, interferon.

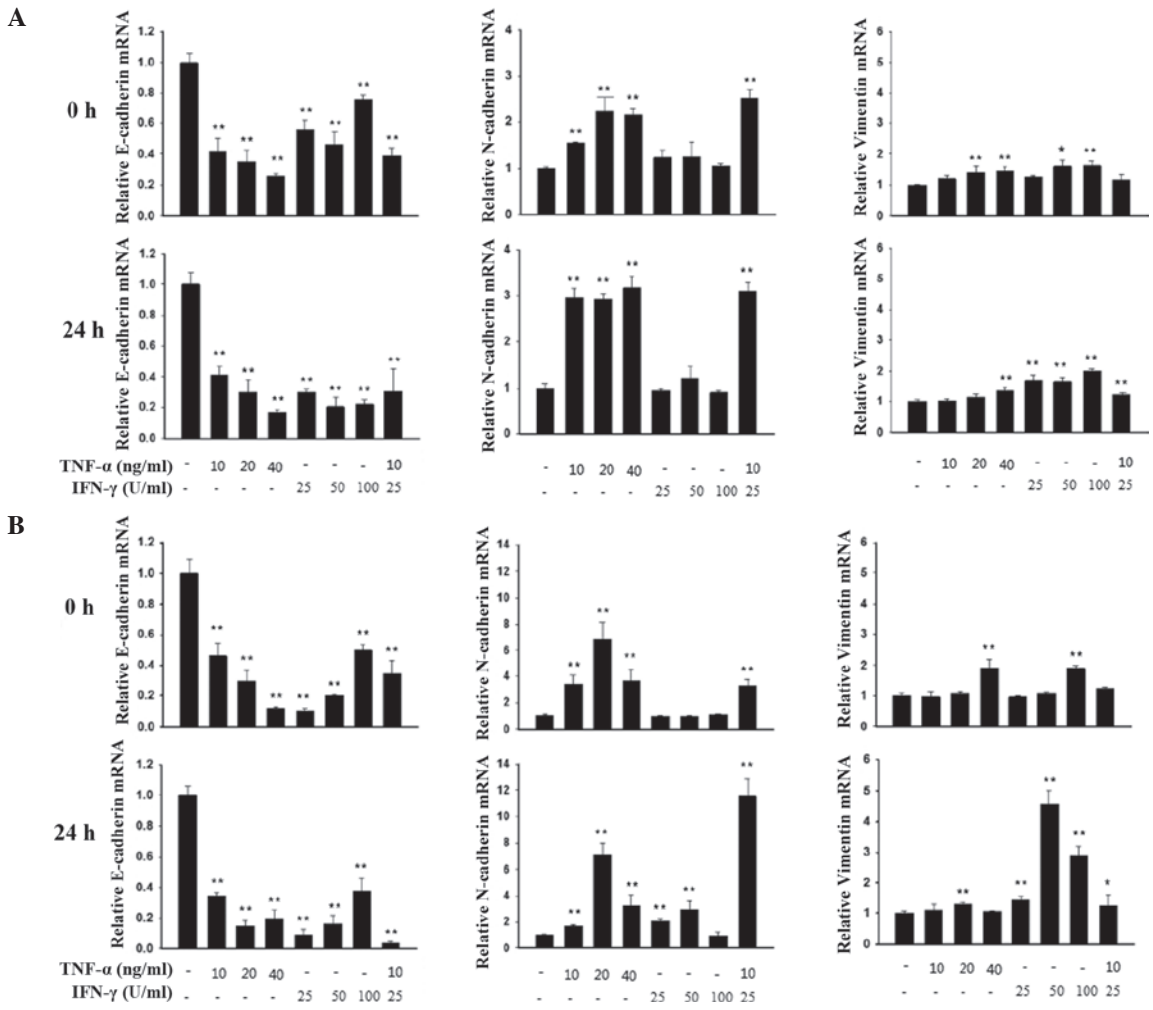

Figure 3. Relative expression of E-cadherin, N-cadherin and vimentin mRNAs in human PTC cell lines, (A) TPC-1 and (B) BCPAP. Expression of EMT genes relative to GAPDH in cells was detected by quantitative polymerase chain reaction, following co-culture with TNF-α and IFN-γ at the indicated time periods and concentrations. The results are expressed as the mean ± standard deviation of three independent experiments (*P<0.05 and **P<0.01, vs. control). PTC, papillary thyroid carcinoma; EMT, epithelial-mesenchymal transition; TNF, tumor necrosis factor; IFN, interferon.

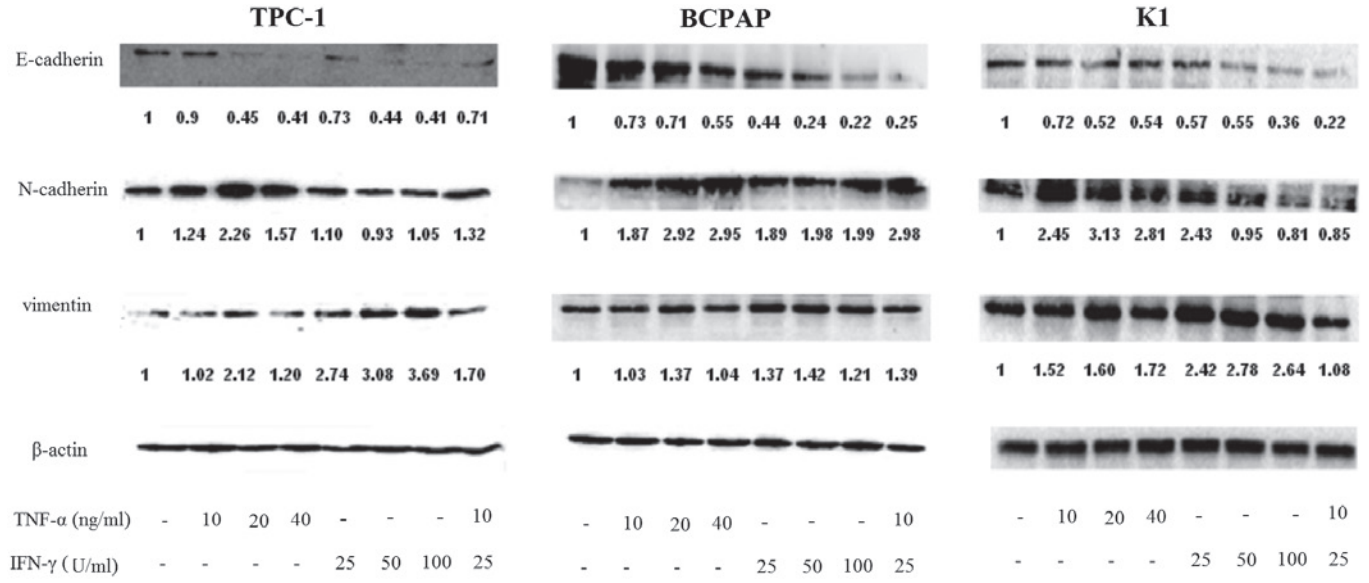

Figure 4. Relative expression levels of E-cadherin, N-cadherin and vimentin proteins in human PTC cell lines. PTC cells were incubated in the presence or absence of TNF-α and IFN-γ for 36 h, and the expression levels of the three EMT makers were analyzed by immunoblot analysis. β-actin serves as the loading control. Data are representative of three independent experiments. PTC, papillary thyroid carcinoma; EMT, epithelial-mesenchymal transition; TNF, tumor necrosis factor; IFN, interferon.

13-fold compared with the control group, while it increased by 10-fold following incubation with IFN-γ. After incubation of K1 cells with TNF-α, the fraction of invasive cells increased by 10-fold compared with the control, while it increased by 6-fold following incubation with IFN-γ. These results suggest that TNF-α and IFN-γ are able to significantly promote the invasiveness of PTC cells.

EMT was induced by TNF-α and IFN-γ in the PTC cell lines. TNF-α-induced EMT has been reported in numerous tumor types; however, EMT induced by IFN-γ alone has rarely been observed (11,22,23). RT-qPCR was used to measure the expression levels of epithelial cell marker, E-cadherin, and the mesenchymal cell markers, N-cadherin and vimentin, in epithelial cell lines, and their expression was used as a measure of EMT.

Epithelial cell lines treated with TNF-α and IFN-γ, alone or in combination, expressed lower levels of E-cadherin mRNA and higher levels of N-cadherin and vimentin mRNA compared with untreated cells (Fig. 3). In TNF-α-treated TPC-1 cells, RT-qPCR revealed that the mRNA expression of E-cadherin was decreased by ~60-70% at 12 and 24 h, while the mRNA expression of N-cadherin was increased by 2-fold at 12 h and 3-fold at 24 h; however, no evident increase was observed in the mRNA expression of vimentin. By contrast, when treated with IFN-γ, the mRNA expression of E-cadherin was decreased by ~20-40% at 12 h and ~70% at 24 h, while that of vimentin was increased by 1.5-fold at 12 and 24 h; however, no evident increase was observed in the mRNA expression of N-cadherin. In BCPAP cells, the observations were similar.

Incubation with low concentrations of TNF-α and IFN-γ was also found to exert a synergistic effect on E-cadherin in TCP-1 and BCPAP cells. TPC-1 cells incubated with a combination of 10 ng/ml TNF-α and 25 U/ml IFN-γ for 12 h expressed 67% more N-cadherin mRNA compared with cells incubated with 10 ng/ml TNF-α alone and 108% more N-cadherin mRNA compared with cells incubated with 25 U/ml IFN-γ alone ($P<0.01$). In addition, BCPAP cells incubated with a combination of 10 ng/ml TNF-α and 25 U/ml IFN-γ for 12 h presented a 6-fold increase in N-cadherin mRNA expression compared with cells incubated with 10 ng/ml TNF-α alone, and a 5-fold increase compared with cells incubated with 25 U/ml IFN-γ alone ($P<0.01$). Although TNF-α and IFN-γ were both able to induce downregulation of E-cadherin mRNA expression, their effect on N-cadherin and vimentin expression differed. TNF-α predominantly upregulated N-cadherin expression, while IFN-γ predominantly upregulated vimentin expression.

These results were further validated by immunoblot analysis (Fig. 4), which indicated that the expression of E-cadherin protein was decreased to varying levels in all three cell lines, when compared with the control groups. E-cadherin protein expression was reduced by 10-59% in TPC-1 cells, by 23-78% in BCPAP cells and by 28-78% in K1 cells. Consistent with the mRNA levels, TNF-α also predominantly induced N-cadherin protein, while IFN-γ predominantly induced vimentin, with detailed results shown in Fig. 4. However, when administered in higher concentrations (40 ng/ml TNF-α and 100 U/ml IFN-γ), the synergistic effect of these cytokines on EMT progression was less pronounced. Upon measuring the protein expression to evaluate the superposition effect of these two cytokines (10 ng/ml TNF-α and 25 U/ml IFN-γ), the EMT progression was unclear, potentially due to the excessively high concentration of cytokine used for an extended period (36 h), which may have induced apoptosis.

Discussion

In the present study, the influence of proinflammatory cytokines TNF-α and IFN-γ on the malignant progression of PTC cell lines *in vitro* was investigated. TNF-α and IFN-γ were found to enhance the capacity of PTC cell lines to migrate and invade, and this process coincided with the downregulation

of E-cadherin and upregulation of N-cadherin and vimentin, which are hallmarks of EMT.

TNF-α and IFN-γ contributed to the metastasis and invasion of three PTC cell lines, including the BCPAP and K1 cell lines that harbor the *BRAF* mutation and the TPC-1 cell line that harbors the *RET*/*PTC* mutation. The three cell lines responded to TNF-α and IFN-γ treatment in a similar manner, indicating that the oncogenic mutation did not influence the response to these cytokines.

In order to investigate the mechanism of EMT initiated by TNF-α and IFN-γ, the present study also examined the expression of various EMT markers. The expression of E-cadherin in untreated PTC cells was found to be high, which is consistent with the findings of previous studies (29). Liu and Brown reported that E-cadherin was expressed in well-differentiated thyroid carcinomas, including papillary and follicular carcinomas, but not in anaplastic thyroid carcinoma (29). E-cadherin was also expressed at a high level in thyroid cells at all development stages (30), indicating that embryonic thyrocytes maintain original epithelial differentiation and homotypic cell-cell adhesion is mediated by E-cadherin. Following treatment with TNF-α and IFN-γ, the expression of E-cadherin mRNA and protein was reduced, which indicated the first observed functional consequence of the EMT process. Reduced E-cadherin expression has also been observed in the WRO follicular thyroid carcinoma cell line during TNF-α stimulation, and E-cadherin expression was restored when TNF-α stimulation ceased (31). Furthermore, in differentiated thyroid carcinomas, reduced E-cadherin expression has been associated with a poor outcome (32).

In the current study, the expression levels of N-cadherin and the mesenchymal maker, vimentin, were also examined. N-cadherin mRNA and protein levels were upregulated following incubation with TNF-α, but no changes were observed in the level of N-cadherin expression following incubation with IFN-γ. Loss of E-cadherin and gain of N-cadherin is defined as 'cadherin switch' and indicates EMT in numerous solid tumors (10,11,13). However, increased expression of N-cadherin is not observed in all cells undergoing EMT. For instance, N-cadherin expression was not significantly increased in the EMT induced by TGF-β treatment in the BCPAP and TPC-1 cell lines, or as a result of endoplasmic reticulum stress in PC Cl3 cells (33). The findings of previous studies support the hypothesis that the process defined as EMT comprises a wide spectrum of changes in epithelial plasticity, indicating that different 'subtypes' of EMT exist, differing in the mechanism of progression towards a mesenchymal phenotype (33,34).

Additionally, expression of vimentin, which was increased following incubation with IFN-γ, was not found to be altered by incubation with TNF-α. A number of studies have demonstrated that vimentin is an important regulator of EMT (35-37), and plays a key role in PTC (17). However, vimentin has to be localized in the perinuclear region to be fully functional, and the present study detected the total vimentin expression, which may not accurately represent the concentration of perinuclear vimentin. Therefore, this methodological limitation restricts the conclusions that can be drawn about the influence of IFN-γ and TNF-α treatment on vimentin activity in PTC cells. The present study was only preliminary and thus the downstream pathway of TNF-α and IFN-γ treatment, as well as any differences in TPC-1 and BCPAP cells following exposure to these cytokines, should be further analyzed.

In conclusion, the current study identified that TNF-α and IFN-γ play an important role in regulating the adhesion and migration of PTC cells. The results strongly implicated the occurrence of EMT in the PTC metastases, which was induced by TNF-α and IFN-γ treatment. These findings highlight the role of the tumor microenvironment and EMT phenol-type in PTC metastases, and recommend further investigation to assist the efforts to define predictive diagnostic models and treatments for PTC with lymph node metastases.

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