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Uptake of high-dose folic acid decreases cell viability and proliferation via JAK/STAT pathway in human prostate cancer cells

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ABSTRACT

Aims: Several studies demonstrated that folic acid (FA) supplementation had some effects on prostate cancer initiation. In this study, the effect of FA concentration was evaluated on proliferation and viability in prostate cancer cells (PCCs). Additionally, we also determined the genes dysregulated by the uptake of a certain amount of FA in prostate cancer.

Methods: Changes in cell viability and proliferation were analyzed in PCC for low-dose (Group-1; 1 µM, 10 µM, 100 µM) and high-dose (Group-2; 1 mM, 10 mM) FA concentrations by Trypan blue staining and MTT assay, respectively. mRNA expression level of *FOLR1, FOLR2 FOLR3, JACK1, STAT3, STAT5/A, STAT5/B, PIAS1, PTPN1*, and *SOCS1* were determined by quantitative real-time polymerase chain reaction.

Results: Cell viability and proliferation were significantly lower than healthy prostate epithelial cells in high-dose FA-treated PCCs. mRNA expressions of *FOLR1, JAK1*, and *STAT3* were significantly upregulated in high-dose FA-treated PCCs compared with the controls. There were no significant alterations in the expression of *FOLR2-3, STAT5A/5B, PIAS1*, and *PTPN1* genes, however, *SOCS1* mRNA expression was significantly lower than the controls.

Conclusions: Low-dose FA showed no effect on cell viability and proliferation, whereas viability and proliferation were decreased by the uptake of high-dose FA that was supposed to stimulate the mRNA expression of *FOLR1* in PCCs. Decreased *SOCS1* and increased *JAK1* and *STAT3* gene expressions implicate the dosage-dependent FA effect on JAK/STAT signaling pathway in prostate cancer.

Introduction

There are three types of folate receptors (FR1-adult form, FR2-fetal form and FR3) that transport folate via endocytosis and are activated by folic acid (FA) in the cell (1). These receptors are encoded by *FOLR1*, *FOLR2* and *FOLR3* genes, respectively (2). FA acts as a cofactor in DNA synthesis, repair, and methylation (3,4). Several reports have indicated that the lack of FA results in epigenetic changes, inefficient DNA synthesis and defective cell proliferation (5-7). Additionally, folate deficiency is involved in various diseases such as neural tube defects, anemia, atherosclerosis, and several types of cancers (6,8-11). However, there are conflicting data regarding the effect of FA in the development of tumors. Kuo et al. (6) pointed out that FA inhibits colon cancer cell proliferation. In contrast, Hansen et al. (12) reported that FA activates JAK/STAT pathway and induces dose-dependent proliferation of FR1-positive HeLa cells. Hyperactivation of STAT transcription factors with FA stimulates hematologic malignancies and solid tumors including breast, lung, liver, head and neck, and stomach cancers (13). Moreover, increased activation of the JAK/STAT signaling is associated with a worse prognosis, increased recurrence, and poor overall survival (13,14).

Prostate cancer is one of the most common causes of cancer deaths in men (3). This type of cancer originates in the gland cells of the prostate. The epithelial cells in the prostate (basal, luminal, and neuroendocrine types) are the possible targets for cancer initiation and progression (15). Several authors have reported that dietary supplementation with vitamins or minerals does not affect tumor formation in the prostate (16,17). However, epidemiological studies demonstrated that while low-dose FA

could prevent prostate cancer, high-dose FA increases the risk of malignancy (18).

How FA induces tumor formation is not currently known. Various signaling pathways that halt cell growth and metastasis in prostate cancer have been elucidated by in vitro and in vivo studies (19,20). However, more studies are needed in a molecular aspect to understand whether FA has any effect on prostate cancer progression.

In addition to FA, several studies have been done to understand the molecular basis of prostate cancer and to improve therapeutic strategies by identifying molecular targets. Among them, PIAS1 (protein inhibitors of activated STAT) is a target that modulates various signaling pathways. It has been reported that PIAS1 expression is elevated in metastatic prostate cancer, and it has a significant role in tumor progression (21). Moreover, it has been demonstrated that overexpression of PTPN1 (protein-tyrosine phosphatase 1B) leads to neuroendocrine differentiation of prostate cancer cells (PCCs) (22) and it was indicated as a promoter of prostatic cell growth (23). Furthermore, *SOCS1* (suppressor of cytokine signaling 1) has also been demonstrated as a dysregulated tumor suppressor gene in prostate cancer and could be used as a prognostic biomarker (24). Additionally, all these proteins regulate JAK/STAT signaling pathway (25-27). However, no evidence has been reported regarding the role of FA on these factors associated with prostate cancer progression.

In this study, the effects of variable FA concentrations on the proliferation and viability of PCCs were evaluated and compared with prostate epithelial cells (PECs) to understand whether FA acts on prostate cancer progression. Moreover, we determined the expression level of potential genes related to FA transport and the JAK/STAT pathway.

Methods

Cell culture

Healthy human PEC line (ATCC® PCS-440-010™) and human PCC line (ATCC® PC3-CRL-1435™) were cultured in RPMI-8226 1640 (Sigma-Aldrich-R8758) including 10% (v/v) FBS (BiochromAG, Germany) and 1% (v/v) gentamicin (Biological Industries, Israel) at 37 °C in 5% CO_2. Two groups [(Group-1; low-dose FA (1 µM, 10 µM, 100 µM) and Group-2; high-dose FA (1 mM, 10 mM)] were established for each cell line.

Preparation of FA solution

FA (Sigma) was diluted in RPMI-8226 1640 (Sigma-Aldrich-R8758) at different concentrations (1 µM, 10 µM, 100 µM, 1 mM, 10 Mm).

Cell viability assay

Trypan blue (Sigma) was used to assess cell viability. It was diluted at 0.8 mM in PBS and mixed with the cells in a 1:1 ratio. In this method, live (viable) and dead (non-viable) cells were counted on a hemocytometer (28).

MTT assay

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue) is a colorimetric assay to measure the metabolic activity of cells, and it was used as an indicator of cell proliferation in the current study. Cell proliferation was estimated by MTT after $3x10^4$ cells in a culture flask were treated with variable concentrations of FA (1 µM, 10 µM, 100 µM, 1 mM, 10 mM) for 24 h.

RNA isolation and cDNA synthesis

Cells were harvested using trypsin/EDTA solution (Sigma Aldrich/T4049) after 24 h. Total RNA was extracted in each group via a High Pure RNA Isolation kit (Roche). cDNAs were synthesized using the RevertAid First Strand cDNA synthesis kit (ThermoFisher). The quality of cDNAs was checked with 2% agarose gel.

Quantitative real-time polymerase chain reaction

Selected genes [*FOLR1* (OMIM: 136430), *FOLR2* (OMIM: 136425), *FOLR3* (OMIM: 602469), *JAK1* (OMIM: 147795), *STAT3* (OMIM: 102582), *STAT5/A* (OMIM: 601511), *STAT5/B* (OMIM: 601512) *PIAS1* (OMIM: 603566), *PTPN1* (OMIM: 176885) and *SOCS1* (OMIM: 603597)] were analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). *ACTB* (OMIM: 102630) was used as an internal control. All the forward and reverse primer sequences were retrieved from the PrimerBank database (https://pga.mgh.harvard.edu/primerbank/). Each qRT-PCR was performed in a 20 µL reaction by using LightCycler® 480 System. To get optimum results, qRT-PCR reactions were performed six times for each gene and condition. mRNA expression levels of *FOLR1-3*, *JAK1*, *STAT3*, *STAT5/A-B*, *PIAS1*, *PTPN1,* and *SOCS1* were determined in FA-treated cells compared to control (untreated PCCs). Mean values were obtained in all groups.

Statistical Analysis

The statistical significance was determined by two-tail Student's t-test in Microsoft Excel. $P<0.05$ was considered significant.

Results

Cell viability was over 85% (between 85-92%) in normal and PCCs in all groups (Figure 1). In Group 1, the cell viability ratio was 89-92%. Cell viability was significant only in 10 µM FA-treated PCCs in Group-1 (Figure 1A). However, cell viability was lower in high-dose FA-treated PCCs compared with PECs (Figure 1B). All other concentrations showed similar viability ratios compared to the no FA-treated group in each PCC line (Figure 1).

In the MTT assay, FA dosage for LD 50 (lethal dose 50) was determined as 10 mM. Additionally, all ratios on the MTT assay were statistically insignificant except 100 µM FA-treated PCC in

Group-1 (Figure 2A). However, cell proliferation was reduced in parallel with the elevation of FA concentration in PCCs compared to PECs (Figure 2B). Overall, cell viability and proliferation were not affected in Group 1, but they were significantly decreased in Group 2.

mRNA expression levels of selected genes were studied in PCCs and controls for 1 µM, 1 mM, and 10 mM FA concentrations by qRT-PCR (Figure 3). *FOLR1* expression was significantly upregulated in PCCs compared to PECs at all FA concentrations (Figure 3A). No significant difference was determined for *FOLR2* and *FOLR3* gene expression (Figure 3A). Additionally, *JAK1* mRNA expression was slightly but significantly increased compared to controls (Figure 3B). However, *STAT3* gene expression was significantly upregulated with increasing concentrations of FA (Figure 3B). No significant difference could be obtained for *STAT5/A* and *STAT5/B* gene expression (Figure 3B). Furthermore, *SOCS1*, an inhibitor of the JAK/STAT signaling pathway, *PTPN1*, an inducer of PCC growth and *PIAS1*, an inhibitor of the activated STAT pathway, were also studied at the mRNA level. Although no significant difference was obtained for *PTPN1* and *PIAS1* gene expression, mRNA expression of *SOCS1* was downregulated with increasing concentrations of FA (Figure 3C).

Discussion

In this study, low-dose FA showed no effect on cell viability and proliferation in PCCs. However, the uptake of high-dose FA decreased cell viability and proliferation in PCCs. Moreover, decreased *SOCS1* and increased *JAK1* and *STAT3* gene expressions indicated a dose-dependent effect of FA on the JAK/STAT signaling pathway in PCCs.

Folate is a substance naturally found in fruits and vegetables. The synthetic form of folate is FA. As a source of folate, FA is used in dietary supplements (29). It is also essential for cell growth and division (6). The low level of FA leads to defects in DNA replication, methylation, and repair in the cell (30,31). Furthermore, excessive FA levels can enhance tumor growth in the colon, polyomavirus middle-T-induced breast, and prostate

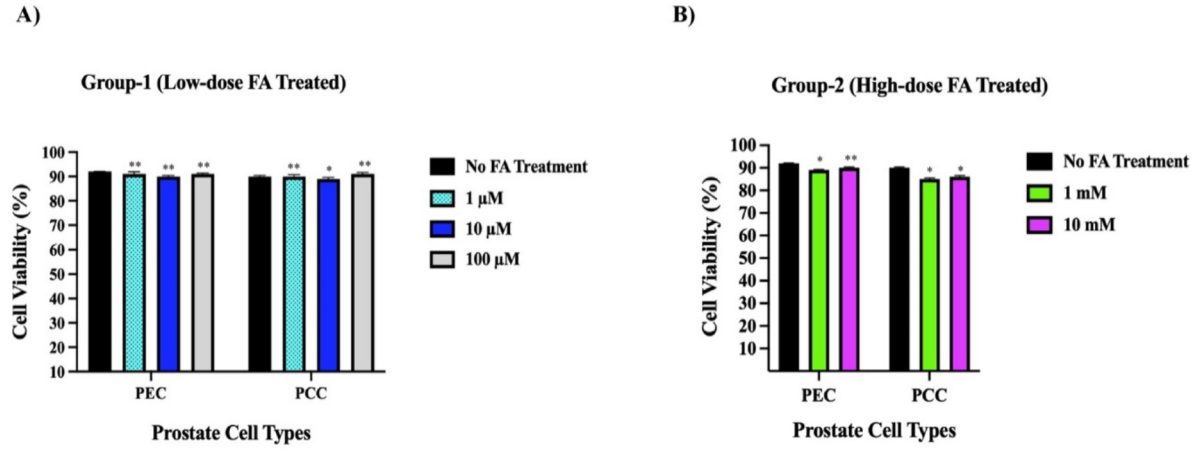

Figure 1. A, B) The effect of variable FA concentrations on the viability of prostate cancer cells
PEC: Prostate epithelial cell, PCC: Prostate cancer cell, FA: Folic acid *p<0.05, **p≥0.05. P values were calculated by comparing untreated and treated PCC

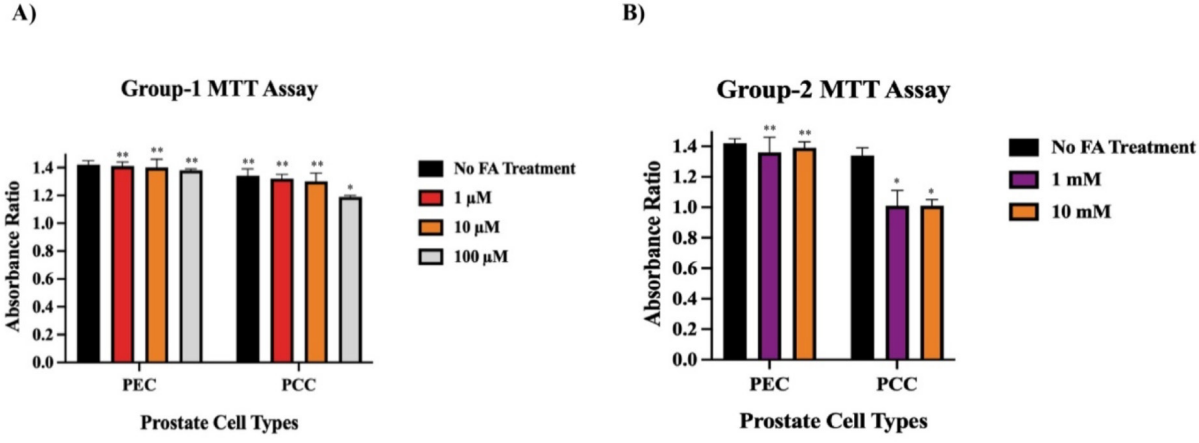

Figure 2. A, B) The results of the MTT assay to estimate prostate cell proliferation after FA treatments
PEC: Prostate epithelial cell, PCC: Prostate cancer cell, FA: Folic acid *p<0.05, **p≥0.05. P values were calculated by comparing untreated and treated PCCs

cancer (32-34). Despite these findings, a few epidemiological studies have shown that FA can prevent prostate cancer at low doses. Therefore, the use of excessive FA may increase the risk of malignancy in the prostate gland (35). The National Cancer Institute of the USA (NCI) describes folate as a protective agent against prostate cancer. Additionally, NCI has also declared FA as a risk factor for prostate cancer when taken at high levels as a supplement (29). Interestingly, for the first time, we demonstrated that excess cellular FA reduced cell proliferation and viability in PCCs.

FRs are overexpressed on the cell surface of solid tumor cells, including ovarian, kidney, lung, brain, endometrial, colorectal, pancreatic, gastric, prostate, testicular, bladder, head and neck, breast, and lung cancer (36). In our study, mRNA expression of *FOLR1* was increased in high-dose FA-treated PCCs compared to controls. In contrast, mRNA expressions of *FOLR2* and *FOLR3* were not significant compared with the controls. It can be suggested that the increasing level of FA concentration causes upregulation of *FOLR1* but not *FOLR2* and *FOLR3* in PCCs. Several factors regulate *FOLR1* expression, such as extracellular folate concentration, intracellular homocysteine concentration, and epigenetic and hormonal regulations (36). Here, we could demonstrate the positive effect of an increase in the level of FA concentration in promoting the expression of *FOLR1* mRNA. Recently, Jia et al. (37) reported that elevated core-fucosylation of FOLR1 can enhance the uptake of folate to the cell to induce epithelial-mesenchymal transition which triggers metastasis and invasion of hepatocellular carcinoma. This finding suggests that FA concentration might not be the only factor to enhance *FOLR1,* but also post-translational modifications of the protein should be considered and PEC-specific glycoproteomic-based studies should be performed.

We identified that mRNA expression of *JAK1* and *STAT3* was upregulated with increasing concentrations of FA compared to controls. However, downregulated *SOCS1* mRNA expression suggests the suppression of this tumor suppressor gene with the elevated level of FA in PCCs. The SOCS family of proteins are negative-feedback inhibitors of signaling induced by cytokines that act via the JAK/STAT pathway (38). Furthermore, SOCS1 acts as a negative regulator of STAT3 (39). These earlier findings confirm our study in which *SOCS1* is downregulated and *STAT3* is upregulated in high-dose FA-treated PCCs.

JAK/STAT pathway, a well-known intracellular signal chain, includes proteins that act on signal transduction. This signaling pathway affects several processes such as cell division, cell death, tumor formation and immunity (40). Additionally, JAK/STAT signaling can change the transcriptional regulation of genes that have a role in cell division (41). Excessive production of STAT proteins has been associated with cancer, in particular aggressive tumor types (42). Groner and von Manstein (43) reported that high-level STAT3 in a cell stimulated *BCL2* and *c-Myc* genes, which are involved in cell division. These findings are inconsistent with our FA-treated PCCs study in which *JAK1 and STAT3* mRNA expressions were upregulated, and cell proliferation was decreased. Furthermore, since SOCS has a role in the inhibition of JAK/STAT signaling (38,42), it can be suggested that decreased level of *SOCS1* is a critical factor to induce JAK/STAT signaling but an unknown mechanism decelerate prostate cancer progression. These findings may pave the way to investigate the effect of high-dose FA on *JAK1* and *STAT3* with further studies.

Study Limitations

The basic limitation of this study is the use of only one type of PCC. More detailed studies should be performed to reveal the precise effect of high-dose FA on prostate cancer.

Conclusion

In conclusion, we demonstrated increased mRNA expression levels of *FOLR1*, *JAK1,* and *STAT3* in PCCs compared to control depending on the increased uptake of FA. These findings emphasize that a higher level of cellular FA might decrease *SOCS1* expression and trigger JAK/STAT signaling by inducing JAK1 and STAT3. FA may play a dual role in prostate carcinogenesis and circulating FA at high concentration might

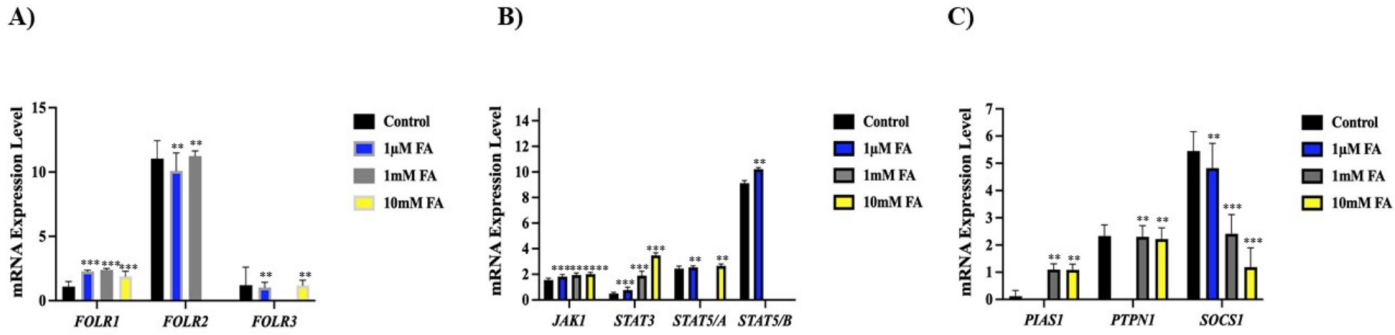

Figure 3. A, B) Gene expression analysis of selected genes
PCC: Prostate cancer cell, FA: Folic acid. Control is the untreated PCC group. **p≥0.05, ***p<0.005. P values were calculated by comparing untreated and treated PCCs. Blank columns represent no obtained data for related genes

enhance prostate cancer progression (44) or high-dose cellular FA may reduce the proliferation of PCCs via dysregulation of JAK/STAT signaling. However, new molecular targets should be identified to define the effect of a higher concentration of FA on the SOCS1/JAK/STAT pathway, which could clarify how PCC proliferation is inhibited.

Ethics

Ethics Committee Approval: This study was approved by the Gülhane Military Medical Academy Ethics Committee (17/11/2014- GATA Ethics Committee decision 2014-Session 46).

Informed Consent: Since it was a study based on a commercial cell line no consent form was obtained.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Ş.G., Z.D.Ç., Concept: Ş.G., Z.D.Ç., Design: Ş.G., Z.D.Ç., Data Collection or Processing: Ş.G., Z.D.Ç., H.G., Analysis or Interpretation: Ş.G., Z.D.Ç., H.G., Literature Search: Ş.G., H.G., Writing: Ş.G., H.G.

Conflict of Interest: No conflict of interest was declared by the authors.

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