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Investigating the Influence of Everolimus, Recombinant TGF-alpha, and Methotrexate on Cell Cycle Phases in Human Cancer Cell Lines

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HIGHLIGHTS

- MTX treatment induced the transition from G1 to S phase in ovarian cancer cell line.
- TGF-alpha treatment decreased the percentage of cells in G1 in ovarian cancer cells.
- TGF-alpha reduced the number of cells in G1 in the triple-negative breast cancer.
- Colorectal cancer cells displayed intrinsic resistance to the treatments.

Abstract: The disruption of cell cycle phases is one of the characteristics acquired by tumor cells during the process of carcinogenesis. In this study, we investigated the differences in cell cycle phases among human cell lines derived from triple-negative (BT-549), colorectal (RKO-AS45-1), and ovarian (TOV-21G) cancers treated with everolimus, recombinant TGF-alpha, and Methotrexate (MTX). Our data showed that MTX induced the transition from G1 to S phase in TOV-21G, while TGF-alpha decreased the percentage of cells in G1. For the triple-negative breast cancer (TNBC) cell line, TGF-alpha reduced the number of cells in G1. In the case of the RKO-AS45-1 cell line, no treatment affected the dynamics of the cell cycle in these cells, indicating intrinsic resistance to the treatments used.

Keywords: everolimus; recombinant tgf-alpha; methotrexate; human cancer cell lines; cell cycle.

INTRODUCTION

The signals that govern cell division are commonly known as the cell cycle, consisting of five distinct phases: quiescence (G0), Gap 1 (G1), DNA replication/synthesis (S), Gap 2 (G2), and mitosis (M). Transitions between these phases are initiated by fluctuating levels of cyclins and cyclin-dependent kinases (cdks). Each cell cycle phase is distinguished by the formation of specific complexes of cyclin/cdk heterodimers [1].

The disruption of cell cycle phases and consequent sustained proliferative signaling are some of the characteristics acquired by tumor cells during the process of carcinogenesis [2]. Barriers against tumor initiation encompass the activation of the DNA damage checkpoint, which subsequently triggers apoptosis or cell cycle arrest [3].

Different chemotherapeutic agents are capable of causing direct DNA damage and disrupting cell division by targeting different phases of the cell cycle. These drugs include platinum derivatives, etoposide, gemcitabine, and methotrexate [4-6].

In a previous study, we evaluated the capacity of everolimus at 100nM as an inducer of Epithelial-to-mesenchymal transition (EMT) in cell lines derived from human breast (BT-549), colorectal (RKO-AS45-1), and ovarian (TOV-21G) cancer. We demonstrated that the EMT markers were differentially expressed after treatment with everolimus. Additionally, we showed that everolimus and TGF-alpha were capable of decreasing the transepithelial/transendothelial electrical resistance in the RKO-AS45-1 cell line [7]. Therefore, this study aimed to investigate the role of everolimus, TGF-alpha, and methotrexate in the cell cycle phases of human cancer cell lines.

MATERIAL AND METHODS

Cell culture

The cell lines BT-549 (triple-negative breast ductal carcinoma - HTB-122 ™), RKO-AS45-1 (colorectal carcinoma - CRL-2579 ™), TOV-21G (ovarian adenocarcinoma - CRL-11730 ™), and WI-26 VA4 (lung fibroblast - CCL-95.1 ™ used as control) were purchased from the American Type Culture Collection (ATCC®), and propagated in a monolayer adherent culture. The BT-549 cell line was cultured in RPMI 1640 Medium supplemented with 10% Fetal Bovine Serum (FBS) inactivated at 56°C for 30 min, 1% 0.2M L-glutamine, and 10mg/ml of Bovine Insulin. The TOV-21G cell line was grown in Dulbecco's Modified Eagle's Medium High Glucose supplemented with 15% FBS and 1% 0.2 M L-glutamine, while the RKO-AS45-1 and WI-26 VA4 cell lines were cultured in RPMI 1640 Medium supplemented with 10% FBS and 1% 0.2 M L-glutamine. Cells were incubated at 37°C with humidified atmosphere enriched with 5% CO2. The experiments were carried out obeying a certain passage number.

Cell cycle analysis

The cell lines were seeded in 24-well plates (BT-549 and WI-26 VA4 – $7.23x10^4$ cells/well, RKO-AS45-1 $14.46x10^4$ cells/well, and TOV-21G – $23.15x10^4$ cells/well) and incubated overnight at 37ºC in 5% CO2. After treatment with everolimus at 100 nM, Methotrexate at 10 nM (Miantrex), and TGF-alpha (Sigma-Aldrich) at 700 ng/mL for 24 h, cell lines in the monolayer were trypsinized, fixed in 70% ethanol for 2h at -20ºC, washed once with 1x PBS, and then labeled with 300nM DAPI (Invitrogen™)/1% Triton X-100 (PlusOne, GE Healthcare) in 1x PBS for 30 min in the dark. The experiments were performed on a FACS Canto II flow cytometer (BD Biosciences, San Jose, CA, USA) with cell acquisition using FACS Diva 6.1.3 software. The data were collected and analyzed using FlowJo software v10.

Data analysis

Statistical analyses were performed with GraphPad Prism 8. Differences in the cell population between the cell cycle phases were determined using non-parametric Kruskal-Wallis with post hoc Dunn’s test. In all cases, p-values < 0.05 were considered statistically significant.

RESULTS

To demonstrate how non-tumoral cells behave during the phases of the cell cycle, we utilized the human fibroblast cell line, WI-26 VA4 (Figure 1 A-D). Our data clearly showed that the majority of cells are in the G1 phase of the cell cycle.

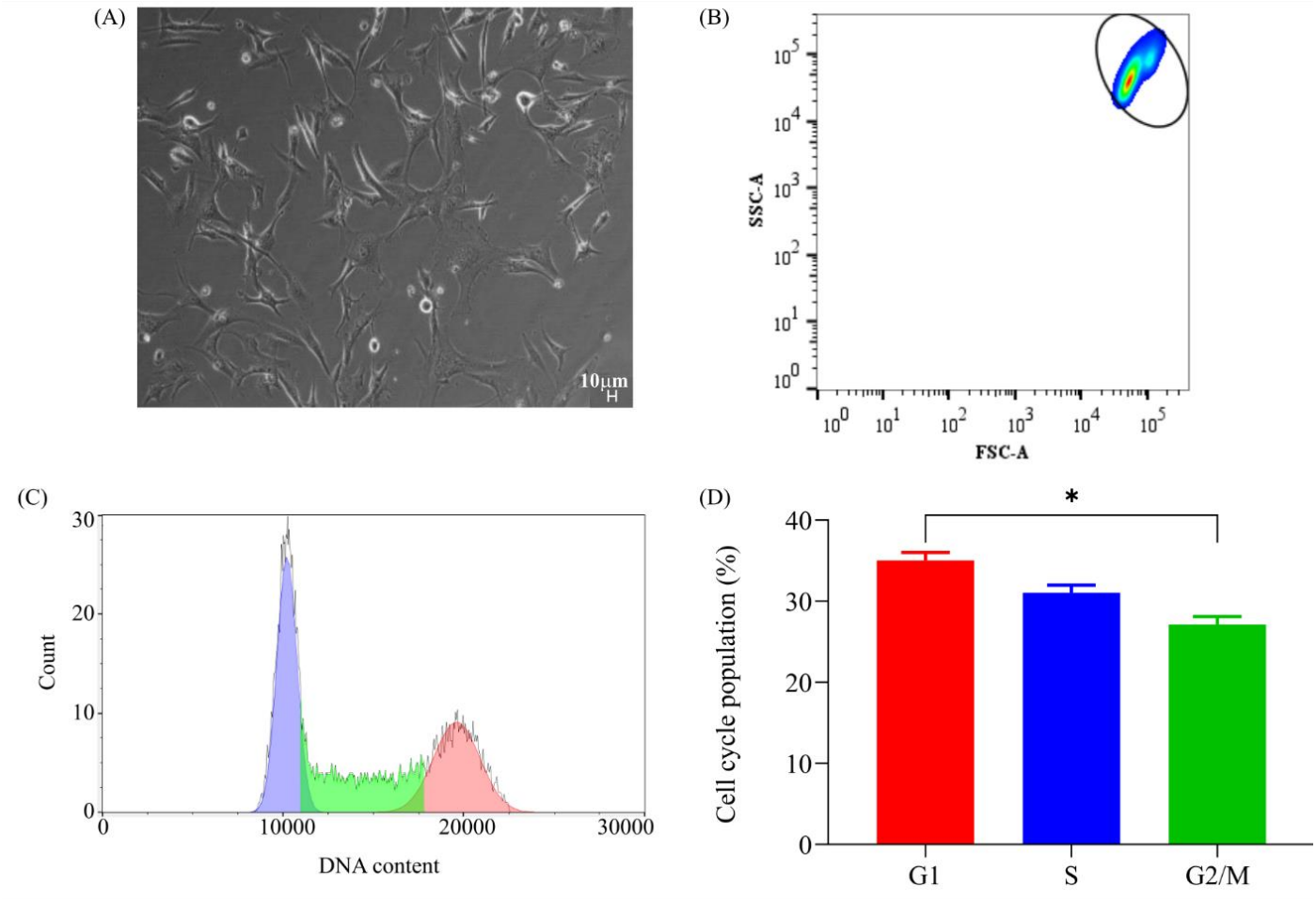

Figure 1. For a non-tumoral cell line, the majority of the cell population is in G1 phase. **(A)** Cell morphology of normal lung fibroblast WI-26 VA4 cells cultured in a monolayer. Representative flow cytometry dot plot **(B)** and histogram **(C)** of untreated WI-26 VA4 cells. Statistical analysis of each cell cycle phase **(D)**. Data were expressed as the mean ± standard deviation (SD). Analysis method: Kruskal-Wallis with post hoc Dunn's test. n= 3. Significance level = p <0.05. (*) difference between groups.

Our results showed that the untreated TOV-21G ovarian cancer cell line had a significantly higher number of cells in the G1 phase compared to the Methotrexate and TGF-alpha groups (Figure 2 A-E and Figure 5 A). Conversely, Methotrexate led to an increase in the population of cells in the S phase compared to the untreated cells. Therefore, Methotrexate predominantly arrested the cell cycle in the S phase.

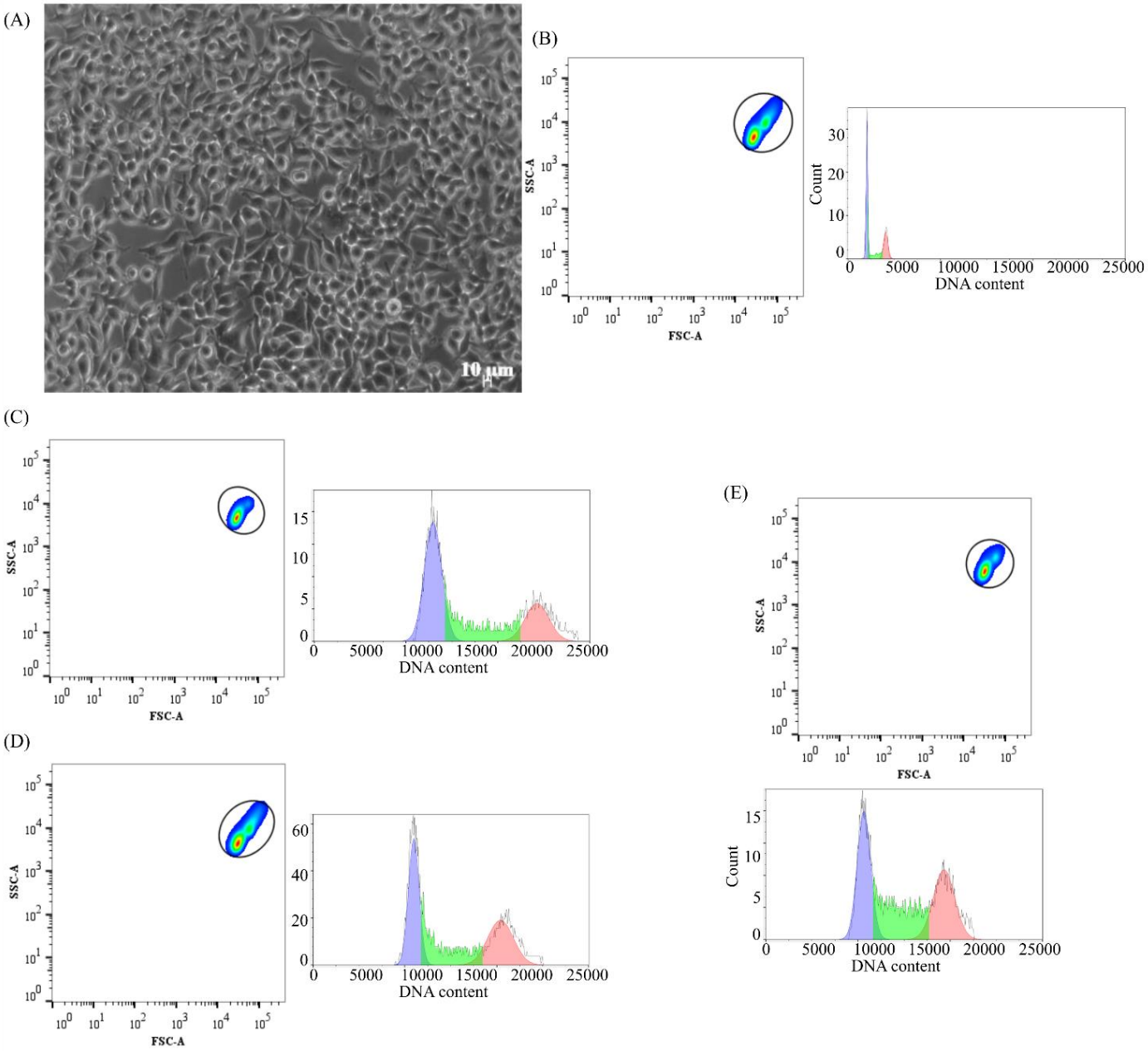

Figure 2. Representative flow cytometry pseudocolor plots and histograms of TOV-21G ovarian cancer cell line. **(A)** Cell morphology of TOV-21G cells cultured in a monolayer. Pseudocolor plots and histograms of untreated cells **(B)**, treated with Everolimus **(C)**, Methotrexate **(D)**, and TGF-alpha **(E)**.

For the triple-negative breast cancer cell line BT-549 (Figure 3 A-E and Figure 5 B), our data showed that treatment with methotrexate was able to reduce the number of cells in the G1 phase. However, treatments with everolimus or TGF-alpha did not impact the percentage of cells in any phase of the cell cycle.

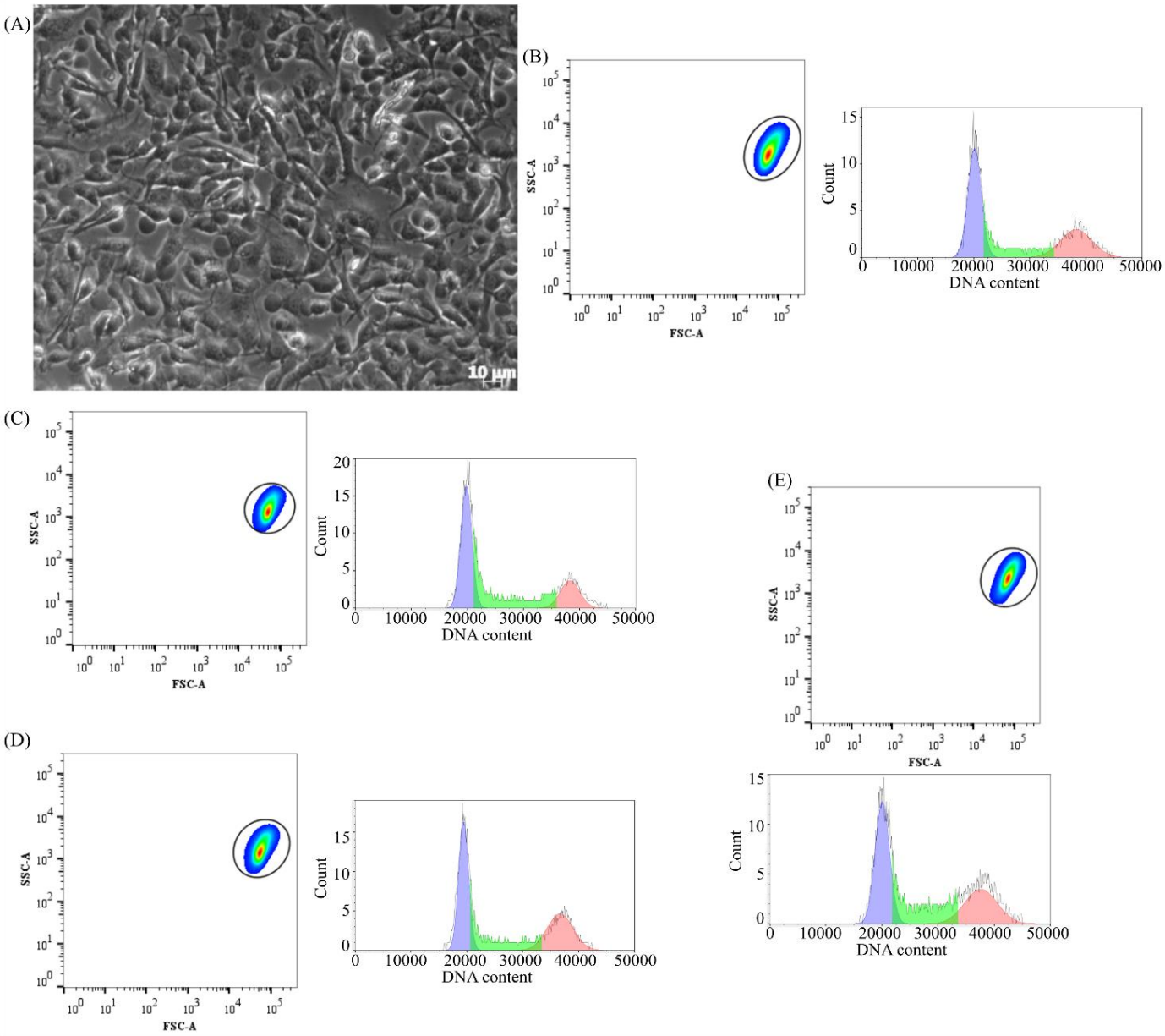

Figure 3. Representative flow cytometry pseudocolor plots and histograms of BT-549 triple-negative breast cancer cell line. **(A)** Cell morphology of BT-549 cells cultured in a monolayer. Pseudocolor plots and histograms of untreated cells **(B)**, treated with Everolimus **(C)**, Methotrexate **(D)**, and TGF-alpha **(E)**.

Regarding the colorectal cancer cell line RKO-AS45-1 (Figure 4 A-E and Figure 5C), no treatment was able to induce any disruption in the phases of the cell cycle.

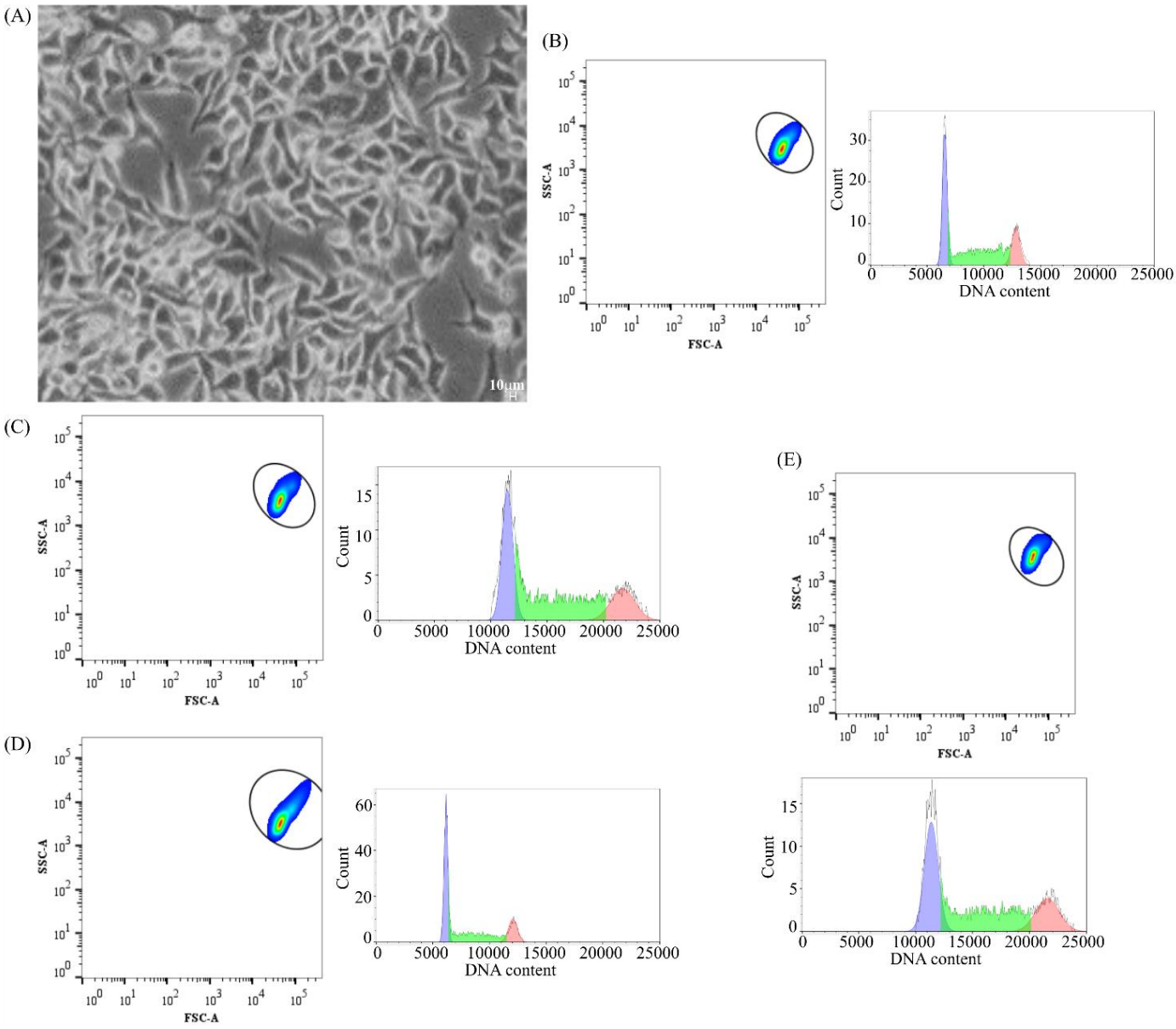

Figure 4. Representative flow cytometry pseudocolor plots and histograms of RKO-AS45-1 colorectal cancer cell line. **(A)** Cell morphology of RKO-AS45-1 cells cultured in a monolayer. Pseudocolor plots and histograms of untreated cells **(B)**, treated with Everolimus **(C)**, Methotrexate **(D)**, and TGF-alpha **(E)**.

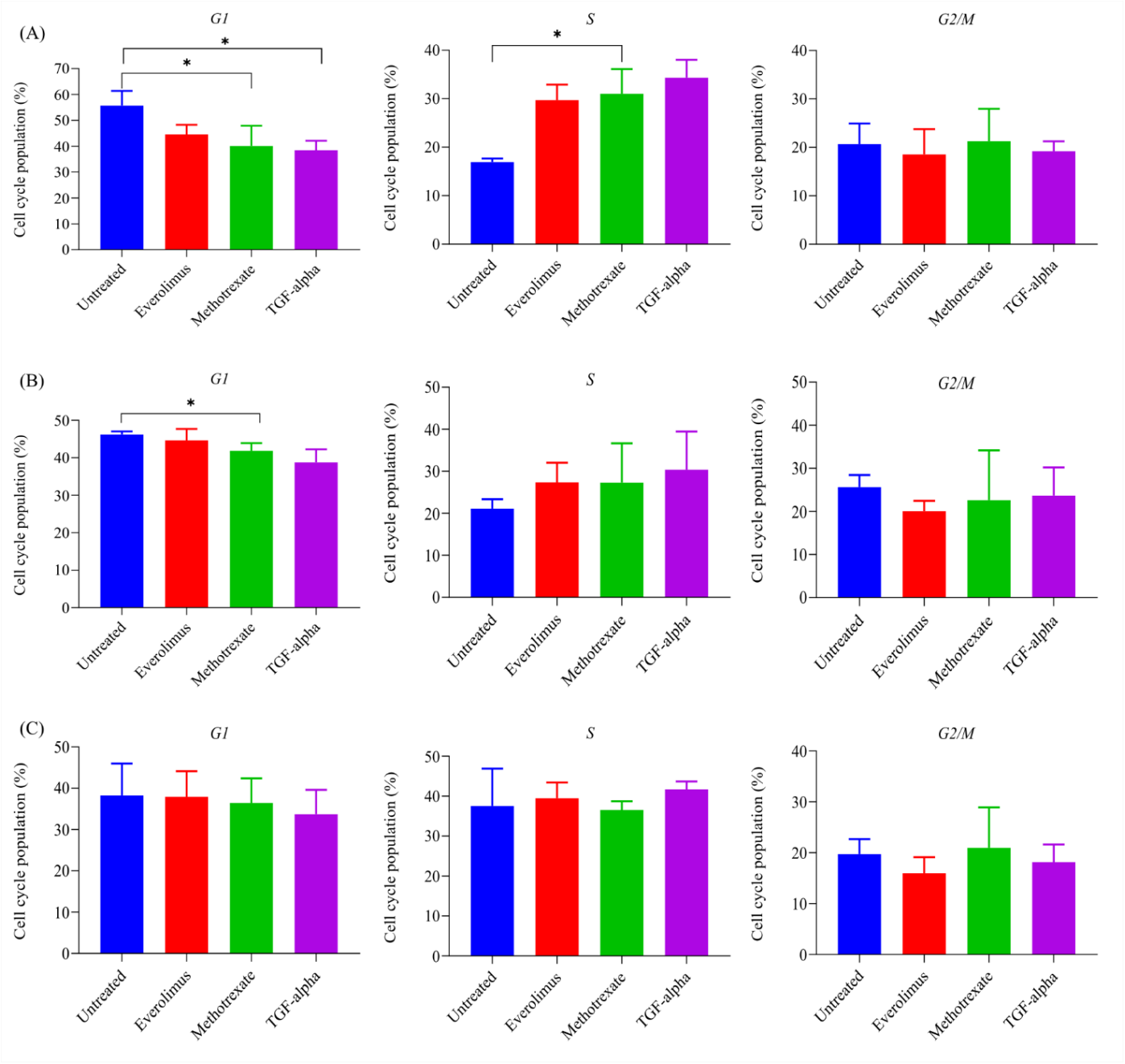

Figure 5. Statistical analysis of cell cycle phases in human tumor cell lines. (A) Methotrexate and TGF-alpha treatments promote changes in the cell cycle of the ovarian cancer cell line (TOV-21G). (B) Methotrexate promotes changes in the cell cycle of the triple-negative breast cancer cell line (BT-549). (C) None of the treatments affected the cell cycle dynamics of colorectal cancer cell line (RKO-AS45-1). Data were expressed as the mean ± standard deviation (SD). Analysis method: Kruskal-Wallis with post hoc Dunn's test. n= 3. Significance level = p <0.05. (*) difference between groups.

DISCUSSION

Cell cycle checkpoints play a crucial role in orchestrating the sequential advancement of the cell cycle, ensuring genomic stability, acting as safeguards against carcinogenesis, and frequently experiencing dysregulation in tumors [1]. In this study, we evaluated whether the antineoplastic agent everolimus or the recombinant protein TGF-alpha affects the dynamics of the cell cycle in human cell lines derived from breast, colorectal, and ovarian cancer. Additionally, we used the chemotherapeutic drug methotrexate as a control, as it is described in the literature to induce alterations in DNA synthesis, thereby disrupting the cell cycle at the S phase [8].

Everolimus is an oral immunosuppressive agent and a specific inhibitor of mTOR (mammalian target of rapamycin), which effectively suppresses cell growth, proliferation, and the transition from G1 phase to S

phase. Additionally, it induces apoptosis by effectively blocking the mTOR signaling pathway [9, 10]. In this study, we showed that treatment with everolimus was not able to promote changes in any of the cell cycle phases in the cell lines derived from human tumors.

In our previous study, we demonstrated that treatment of the same three cell lines used in this study with everolimus upregulated EMT markers. Additionally, everolimus increased the expression of the long non-coding RNA (lncRNA) HOTAIR in BT-549 [7]. The involvement of HOTAIR in modulating drug resistance mechanisms has been extensively documented in various solid tumors [11, 12]. Recent findings have demonstrated that silencing of HOTAIR suppresses cell proliferation, promotes apoptosis, and inhibits cell cycle progression of retinoblastoma cell lines, specifically in the G0/G1 phase [13]. Furthermore, the involvement of HOTAIR in the cell cycle has also been implicated in glioblastoma [14].

Analysis of human cancers at the genetic level has uncovered the widespread inactivation of proteins involved in the G1/S checkpoint, indicating that disruptions in the DNA damage checkpoint are often responsible for the resistance of tumor cells to chemotherapy agents or radiation. Conversely, alterations affecting the G2/M checkpoint are less commonly observed [14].

Methotrexate acts as an inhibitor of dihydrofolate reductase (DHFR), the enzyme that catalyzes the NADPH-dependent conversion of dihydrofolate (FH2) to tetrahydrofolate (FH4). This conversion is a vital step in the synthesis of the purine ring and thymidylate. Previous studies have shown that at low doses, Methotrexate is capable of promoting the transition of cells from G1 to S phase in the cell cycle [15]. Our data demonstrated that treatment with Methotrexate in the ovarian cancer cell line TOV-21G was able to decrease the percentage of cells in the G1 phase and increase the percentage of cells in S phase. Thus, MTX proved to be capable of disrupting the cell cycle dynamics for this cell line.

CONCLUSION

In conclusion, our study investigated the impact of everolimus, recombinant TGF-alpha, and Methotrexate (MTX) on the cell cycle phases of human cell lines derived from triple-negative (BT-549), colorectal (RKO-AS45-1), and ovarian (TOV-21G) cancers. We found that the disruption of cell cycle phases is a characteristic acquired by tumor cells during carcinogenesis. Specifically, MTX induced a transition from G1 to S phase in the TOV-21G ovarian cancer cell line, while TGF-alpha decreased the percentage of cells in G1 in both the TOV-21G and triple-negative breast cancer (TNBC) cell lines. Notably, the RKO-AS45-1 cell line displayed intrinsic resistance, as none of the treatments affected its cell cycle dynamics. These findings highlight the importance of understanding the unique responses of different cancer cell lines to targeted therapies, which may have implications for the development of more effective treatment strategies tailored to specific cancer types.

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Conflicts of Interest: The authors declare that they have no competing interests.

REFERENCES

1. Bower JJ, Vance LD, Psioda M, Smith-Roe SL, Simpson DA, Ibrahim JG, et al. Patterns of cell cycle checkpoint deregulation associated with intrinsic molecular subtypes of human breast cancer cells. NPJ Breast Cancer. 2017;3:9.
2. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646-74.
3. Yao Y, Dai W. Genomic Instability and Cancer. J Carcinog Mutagen. 2014;5.
4. Perez Goncalves BO, Dos Santos GSP, de Andrade WP, Fialho SL, Gomes DA, Silva LM. Phenotypic changes on central nervous system (CNS) tumor cell lines cultured in vitro 2D and 3D models and treated with cisplatin. Acta Histochem. 2021;123(6):151768.
5. Reinhold WC, Thomas A, Pommier Y. DNA-Targeted Precision Medicine; Have we Been Caught Sleeping? Trends Cancer. 2017;3(1):2-6.
6. Wu KF, Liang WC, Feng L, Pang JX, Waye MM, Zhang JF, et al. H19 mediates methotrexate resistance in colorectal cancer through activating Wnt/beta-catenin pathway. Exp Cell Res. 2017;350(2):312-7.
7. Goncalves BOP, De Andrade WP, Da Conceicao Braga L, Fialho SL, Silva LM. Epithelial-to-mesenchymal transition markers are differentially expressed in epithelial cancer cell lines after everolimus treatment. Oncol Lett. 2020;20(5):158.
8. Ernst P, Killmann SA. Perturbation of generation cycle of human leukemic myeloblasts in vivo by methotrexate. Blood. 1971;38(6):689-705.
9. Chen G, Ding XF, Bouamar H, Pressley K, Sun LZ. Everolimus induces G(1) cell cycle arrest through autophagy-mediated protein degradation of cyclin D1 in breast cancer cells. Am J Physiol Cell Physiol. 2019;317(2):C244-C52.

10. Petrossian K, Nguyen D, Lo C, Kanaya N, Somlo G, Cui YX, et al. Use of dual mTOR inhibitor MLN0128 against everolimus-resistant breast cancer. Breast Cancer Res Treat. 2018;170(3):499-506.
11. Goncalves BOP, Fialho SL, Silvestrini BR, Sena IFG, Dos Santos GSP, Assis Gomes D, et al. Central nervous system (CNS) tumor cell heterogeneity contributes to differential platinum-based response in an in vitro 2D and 3D cell culture approach. Exp Mol Pathol. 2020;116:104520.
12. Botti G, Collina F, Scognamiglio G, Aquino G, Cerrone M, Liguori G, et al. LncRNA HOTAIR Polymorphisms Association with Cancer Susceptibility in Different Tumor Types. Curr Drug Targets. 2018;19(10):1220-6.
13. Fu K, Zhang K, Zhang X. LncRNA HOTAIR facilitates proliferation and represses apoptosis of retinoblastoma cells through the miR-20b-5p/RRM2/PI3K/AKT axis. Orphanet J Rare Dis. 2022;17(1):119.
14. Zhao J, Jin W, Yi K, Wang Q, Zhou J, Tan Y, et al. Combination LSD1 and HOTAIR-EZH2 inhibition disrupts cell cycle processes and induces apoptosis in glioblastoma cells. Pharmacol Res. 2021;171:105764.
15. Cipolleschi MG, Marzi I, Rovida E, Olivotto M, Dello Sbarba P. Low-dose methotrexate enhances cycling of highly anaplastic cancer cells. Cell Cycle. 2017;16(3):280-5.