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RESEARCH ARTICLE

Phenethyl isothiocyanate Regulates the Cancer Stem Cell Phenotype of SNU449 Hepatocellular Carcinoma Cells via STAT3-CD44 Axis

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SUMMARY

Cancer stem cells play an essential role in resistance to therapy, invasion, metastasis, and recurrence. CD44 is one of the well-known surface markers for hepatocellular carcinoma, and its expression level is related to poor survival and a high recurrence rate. The effect of phenethyl isothiocyanate (PEITC) on SNU449 hepatocellular carcinoma cell line cancer stem cells is not known. The goal of the present study was to investigate whether PEITC regulates the cancer stem cell phenotype of SNU449 cells. Cell viability, colony formation, and wound healing assays were performed to determine proliferative and migratory characteristics. Caspase 3, CD44, Akt/mTOR, and p38/STAT3 protein expression levels were measured by Western blotting. Compared to control confluence, gap fill, and migration rate were increased while half gap time was decreased in PEITC-treated cells. Compared to control-treated cells, CD44 (3.2-fold) and p-STAT3 (2.44-fold) protein expressions were upregulated in PEITC-treated cells. Results of this study suggest that STAT3-mediated upregulation of CD44 leads to the gain of cancer stem cell phenotype of PEITC-treated SNU449 cells.

Key Words: *Phenethyl isothiocyanate, cancer stem cell, hepatocellular carcinoma*

Fenetil izotiyosiyanat SNU449 hepatoselüler karsinom hücrelerinin kanser kök hücre fenotipini STAT3-CD44 yolu ile düzenler

ÖZ

Kanser kök hücreleri tedavi direnci, invazyon, metastaz ve nükste önemli rol oynar. Hepatoselüler karsinom için bilinen yüzey belirteçlerinden biri olan CD44' ün ekspresyon seviyesi hayatta kalma süresinin kısalması ve yüksek nüks oranı ile ilişkilidir. Fenetil izotiyosiyanatın (PEITC) SNU449 hepatoselüler karsinom hücre hattı kanser kök hücreleri üzerindeki etkisi bilinmemektedir. Bu çalışmanın amacı, PEITC'nin SNU449 hücrelerinin kanser kök hücre fenotipini nasıl düzenlediğini araştırmaktır. Çoğalma ve göç özelliklerini belirlemek için hücre canlılık, koloni oluşum ve yara iyileşme testleri gerçekleştirilmiştir. Kaspaz 3, CD44, Akt/mTOR ve p38/STAT3 protein ekspresyonları Western blotlama ile ölçülmüştür. PEITC uygulanan hücrelerde hücre yoğunluğu, boşluk doldurma ve göç hızı artarken, yarı boşluk zamanının azaldığı bulunmuştur. PEITC uygulanan hücrelerde, CD44 protein seviyesi 3.2 kat ve p-STAT3 protein seviyesi 2.4 kat daha yüksek bulunmuştur. Bu çalışmanın sonuçları, PEITC uygulanan SNU449 hücrelerinin kanser kök hücre fenotipini kazanmasına STAT3-aracılı olarak artan CD44 seviyelerinin neden olduğunu göstermektedir.

Anahtar Kelimeler: *Fenetil izotiyosiyanat, kanser kök hücre, hepatoselüler karsinoma*

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INTRODUCTION

Liver cancer ranks 6th among the malignancies of the individuals; however, it ranks 3rd among the cancer-related deaths worldwide after lung and colorectal cancers (Sung et al., 2021). Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer, and chronic liver disease, and cirrhosis are the two major risk factors. HCC accounts for 75-85% of global cases and is followed by cholangiocarcinoma (Sayiner, Golabi, & Younossi, 2019; Sung et al., 2021). HCC is an invasive tumor that grows rapidly, infiltrates blood vessels, and spreads to distant organs via the bloodstream (B. Yang et al., 2018).

Accumulating body of evidence suggests that resistance to chemo- and radiotherapy, invasion, metastasis, and recurrence can be attributed to cancer stem cells (CSCs) and it has been demonstrated in many tumors such as pancreatic, colon, gastric, and glioblastoma (Askan et al., 2021; X. P. Xie et al., 2022; Yao et al., 2020; Zhang, Wang, Lu, Zhang, & Zheng, 2019). Non-CSCs cancer cells do not have the ability of self-renewal and differentiation properties; CSCs are prerequisites for the formation, progression, and maintenance of tumor phenotype (Ayob & Ramasamy, 2018). Conventional cancer treatment focuses mainly on reducing the tumor bulk, and it does not explicitly target the CSC in the tumors. For this reason, the efficacy of conventional chemotherapy has been limited (Song et al., 2021). CSCs and related pathways must be included in the list of targets to attain the desirable results.

Cluster of differentiation 44 (CD44), one of the most essential CSC markers, along with CD133 and epithelial cell adhesion molecule are highly specific markers for HCC (Koyama et al., 2020). CD44 is a glycoprotein that is located on the surface of the cells and it takes part in cell-cell interaction and adhesion. In HCC, CD44 expression has been reported to be overexpressed and patients who have upregulated expression of CD44 showed significantly poor overall survival and cumulative recurrence risk (P. Xie et al., 2022). The expression level of CD44 can be regulated through signaling pathways, including phosphatidylinositol-3-kinase/Akt serine threonine kinase/mechanistic target of rapamycin (PI3K/Akt/mTOR) and Janus kinase/Signal transducer and activator of transcription 3 (JAK/STAT3). CD44 levels have been reduced by blocking the PI3K/Akt/mTOR pathway with mTOR inhibitors, while the activation of the STAT3 pathway has resulted in the expansion of CD44-positive HCC CSCs (Kim, Jiang, Badawi, & Schmittgen, 2017; Wan et al., 2014).

Glucosinolates are a well-known dietary component of cruciferous vegetables such as watercress and broccoli, and they are a rich source of sulfur-containing compounds. Upon hydrolysis, glucosinolates give a variety of isothiocyanates, like phenethyl isothiocyanate (PEITC) (Yun et al., 2017). PEITC exerts its cancer-chemopreventive effect mainly by inducing the expression of Phase II drug-metabolizing enzyme, which results in the rapid elimination of xenobiotics, cell cycle arrest, and apoptosis (Shin, Lim, Cho, & Nho, 2021)However, the impact of PEITC on CSC phenotype in the Human hepatocellular carcinoma cell line SNU449 HCC cell line has not been investigated.

In the present study, the impact of PEITC on CSCs has been evaluated by examining proliferative capacity, migratory characteristics, and protein expression of CD44, mTOR, and STAT3 pathways.

MATERIAL and METHODS

Cell culture

Human hepatocellular carcinoma cell line SNU449 from American Type Tissue Collection (ATCC, CRL-2234) was cultured in Roswell Park Memorial Institute 1640 Medium (RPMI-1640, Sigma) medium containing 10% heat-inactivated Fetal Bovine Serum (FBS, Sigma) and 1% Penicillin-Streptomycin-Neomycin (Sigma) at 37 °C with 5% CO_2 under a humidified atmosphere.

Cell viability assay

SNU449 cells were resuspended in RPMI-1640 and seeded at a density of 10000 cells/well in 96-well cell culture plate, incubated overnight, and treated

with the serial concentrations of cisplatin (Kocak Farma, Turkey) (1, 2, 5, 10, 20, 50, 100, and 200 µg/mL) and PEITC (Sigma;) (1, 2, 5, 10, 20, 50, 100, 200, 500, and 1000 µg/mL) in RPMI-1640 for 24 h. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma) solution (5 mg/mL in phosphate-buffered saline (PBS) was added to each well and incubated for four h. In the following step, 100 µL dimethyl sulfoxide (DMSO, Merck) was added and shaken for 10 min to dissolve purple formazan crystal. Then, absorbance was measured at 570 nm using a microplate reader (Biotek, Synergy H1m), and median inhibition concentration (IC50) was calculated.

Colony formation assay

SNU449 were seeded to 6-well plates at a density of 1000 cells/well, incubated overnight, and treated with cisplatin and PEITC, 50 µg/mL and 200 µg/mL, respectively, for 24 h. Then, the medium was replaced with fresh RPMI-1640 and changed every 2-3 days. Cultures were washed with PBS and, cell colonies were fixed with methanol:acetic acid (3:1) for 5 min, stained with 0.5% crystal violet in methanol for 15 min, and washed with water. Colonies counted under a microscope (Leica, DMi8), plate efficiency (PE), and surviving fraction (SF) were calculated with the following formulas.

PE = (number of colonies formed/number of cells seeded)*100

SF = (PE of cisplatin or PEITC-treated cells/PE of control cells)*100

Wound healing assay

SNU449 cells were seeded in a 60 mm cell culture petri dish and cultured until 80% confluence. The cell monolayer was scratched with a sterile 100 µL pipette tip and washed twice with PBS to remove cell debris. Cells were treated with 50 µg/mL cisplatin or 200 µg/mL PEITC for 24 h and, scratch images were captured at specified time points using a cell imager (Leica, Paula). Confluence, gap fill, migration rate, and half gap time values at each time point were obtained from the software of the cell imager.

Western blotting

After treating cells with cisplatin or PEITC, the medium was removed, cells were washed with ice-cold PBS twice, and the wash buffer was removed. Ice-cold radioimmunoprecipitation assay buffer (RIPA, Sigma) containing 1% protease and phosphatase inhibitors (Sigma) was added and incubated at 4 °C for 5 min. The cell culture flask was scraped to remove and lyse residual cells, collected in a microcentrifuge tube, and kept at 4 °C for 30 min. Tubes were centrifuged at 16000xg at 4 °C for 20 min and, supernatant was collected. Total protein concentrations were measured by using a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, catalog no. 23225). Equal amounts of protein were loaded onto 4–20% Mini-PROTEAN® TGX™ Precast Protein Gel (Bio-Rad, 4561093) and transferred onto polyvinylidene fluoride (PVDF) membrane by using Trans-Blot Turbo RTA Mini 0.2 µm PVDF Transfer Kit (Bio-Rad, 1704272) and Trans-Blot® Turbo™ Transfer System (Bio-Rad, catalog no. 1704150). After blocking PVDF membranes with EveryBlot (Bio-Rad, catalog no. 12010020) at room temperature for 5 min, membranes were probed with primary antibodies overnight at 4 °C. Akt (Cell Signaling, catalog no. 4691), p-Akt (Cell Signaling, catalog no. 13038), mTOR (Cell Signaling, catalog no. 2983), p-mTOR (Cell Signaling, catalog no. 5536), p38 (Cell Signaling, catalog no. 8690), p-p38 (Cell Signaling, catalog no. 4631), STAT3 (Cell Signaling, catalog no. 8768), p-STAT3 (Cell Signaling, catalog no. 9145), Caspase3 (Cell Signaling, catalog no. 14220), Cleaved Caspase3 (Cell Signaling, catalog no. 9664), CD44 (Santa Cruz, catalog no. sc7297) and β-Actin (Cell Signaling, catalog no. 4970). Then, incubated with HRP-conjugated secondary antibodies, Anti-rabbit IgG, HRP-linked Antibody (Cell Signaling, catalog no. 7404) or m-IgGκ BP-HRP (Santa Cruz, catalog no. sc516102) at room temperature for 1 h. Bands were visualized by using a Clarity Max Western ECL Substrate (Bio-Rad, catalog no. 1705062) and gel imaging system (Bio-Rad, ChemiDoc) and, band quantification was performed using the Bio-Rad Image Lab software Version 6.1.0 build 7.

Statistical analysis

Kolmogorov-Smirnov test was used for the normality tests of the continuous variables. The continuous variables were expressed as median (IQR). The protein expressions were given as fold changes concerning the untreated control. The continuous variables were compared using a pairwise Kruskal-Wallis test (multiple groups). Any p-value less than 0.05 was considered as significant. All statistical analyses were performed using Statistical Software Package for Social Sciences version 27 (SPSS27, IBM).

RESULTS and DISCUSSION

The Determination of Cisplatin and PEITC Cytotoxicity in SNU449 Cell Line

MTT assay was performed to determine the effect of cisplatin and PEITC on SNU449 cell viability. A concentration-dependent viability decrease was observed. For cisplatin, the cell viability at 20 µg/mL, 50 µg/mL, and 100 µg/mL was found to be 69.61%, 40.08%, and 17.47%, respectively. For PEITC, the cell viability at 200 µg/mL, 500 µg/mL, and 1000 µg/mL was found to be 52.38%, 30.49%, and 25.46%, respectively (Figure 1).

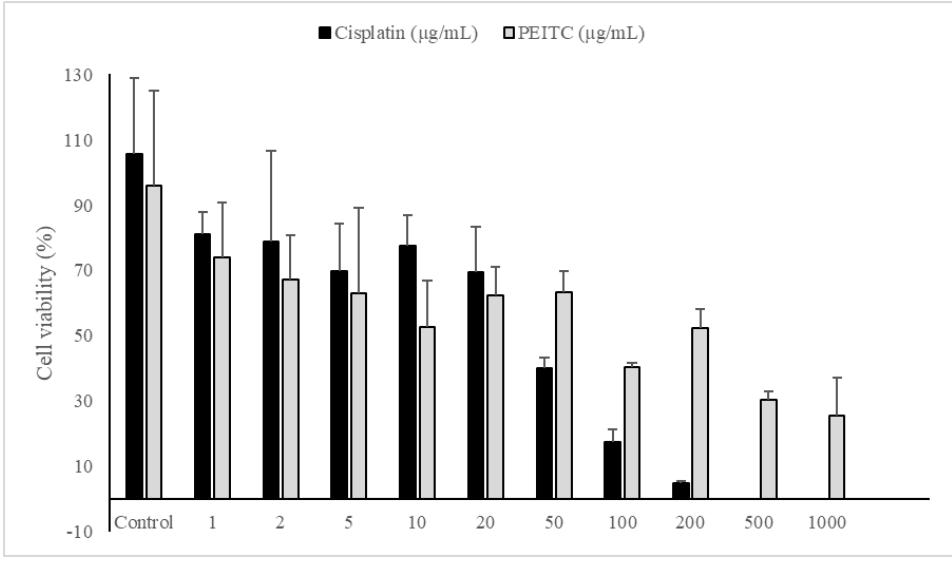

Figure 1. Cell viability of cisplatin and PEITC-treated SNU449 cells was determined by MTT assay. Results were expressed as median ± interquartile range.

IC50 values were calculated by plotting cell viability versus concentration; 200 µg/mL for PEITC and 50 µg/mL for cisplatin were selected as treatment doses. At the end of 24 h exposure to PEITC and cisplatin with treatment dose, the morphology of SNU449 cells was observed under an inverted light microscope and subsequently photographed, as shown in Figure 2.

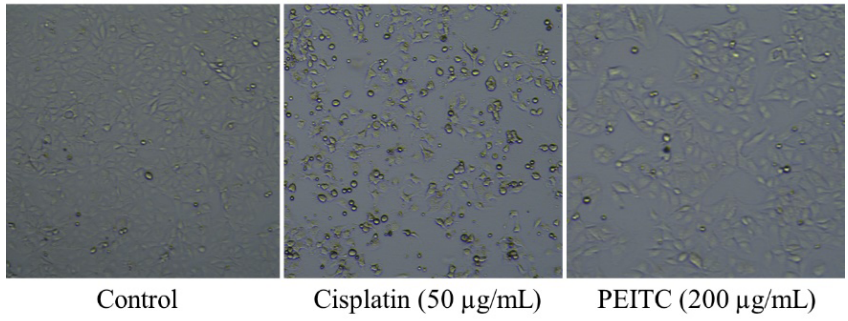

Figure 2. The morphology of SNU449 cells treated with cisplatin and PEITC at the end of 24 h treatment.

PEITC Affects the Proliferative Capacity of SNU449 Cells

The colony formation ability of SNU449 cells treated with PEITC and cisplatin was determined by implementing a colony formation assay. Plate efficiency and survival fraction for PEITC and cisplatin were calculated based on the number of colonies formed (Figure 3). For PEITC and cisplatin, plate efficiencies were found to be 2.7% and 0%, and survival fractions were found to be 21.77% and 0%, respectively.

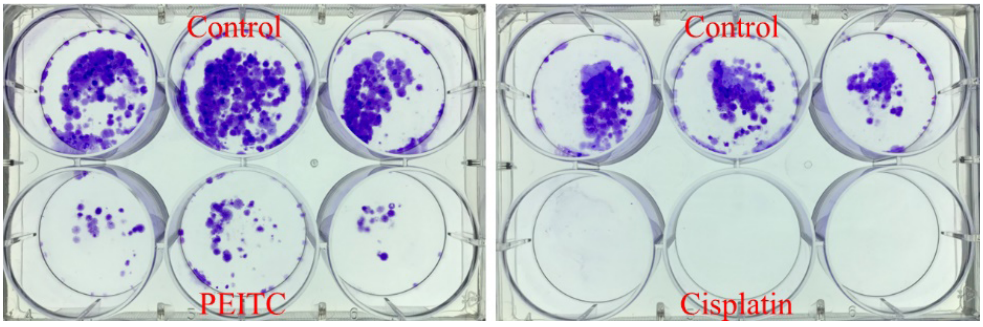

Figure 3. The proliferative capacity of SNU449 cells was evaluated by counting the formed cell colonies at the end of 24h treatment with cisplatin and PEITC.

PEITC Promotes the Migratory Characteristics of SNU449 Cells

The results of the Wound healing assay were summarised in Figure 4a. Confluence was 17.6%, 1.7%, and 31.75%; gap fill (μm^2) was 107.49, 28.3, and 247.72 (Figure 4b); migration rate (%/h) was 1.5, -0.1, and 1.6, and migration rate (μm/h) were 4.4, -0.2, and 5.5 (Figure 4c), for control, cisplatin, and PEITC, respectively. Half gap time (h) were 41.03 and 27.97 for control and PEITC.

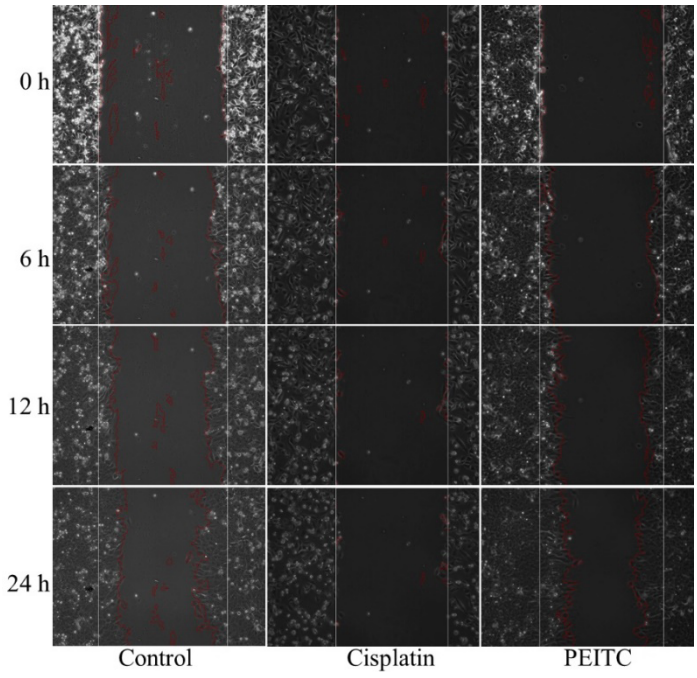

Figure 4a.

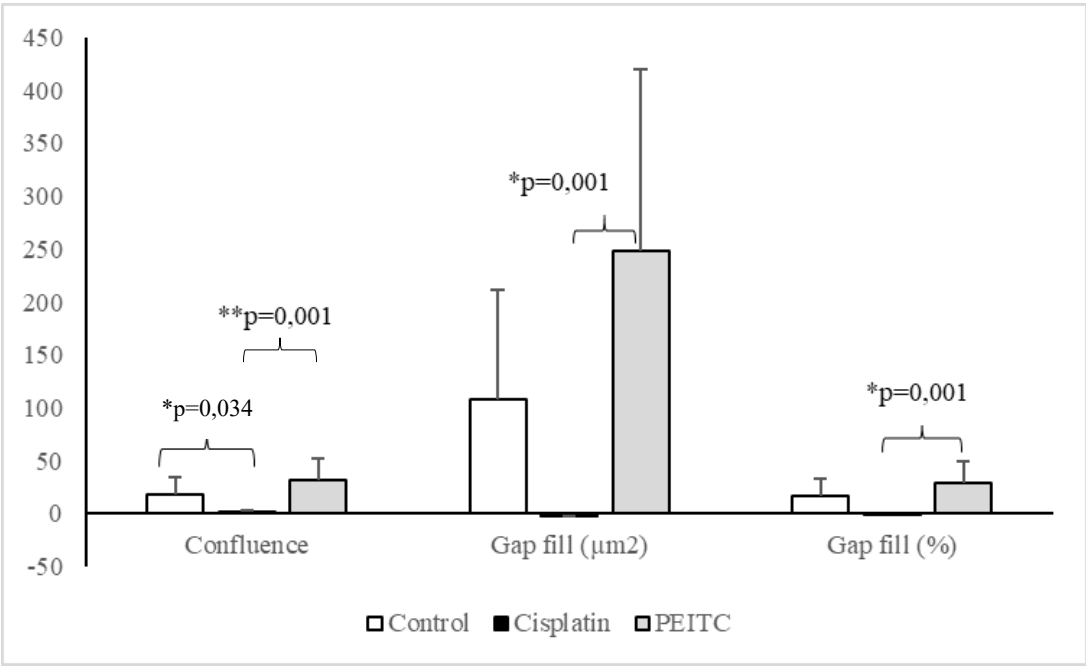

Figure 4b.

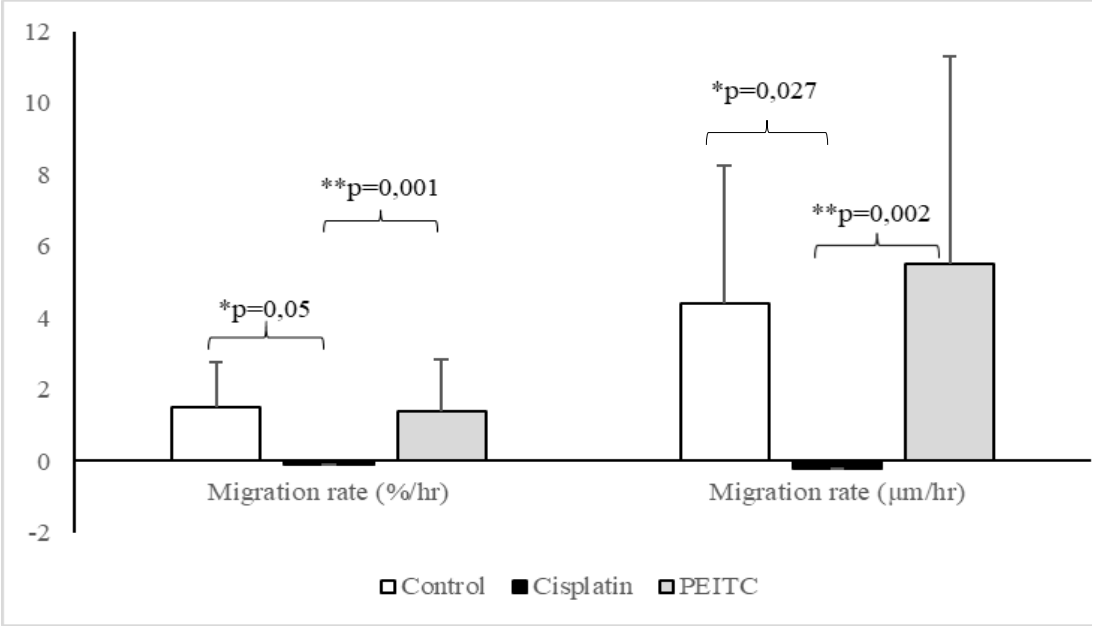

Figure 4c.

Figure 4. At the end of 24h treatment with Cisplatin and PEITC, migratory characteristics of SNU449 cells were evaluated by wound healing assay (Figure 4a). Following scratch at the initial time point, the migration of SNU449 cells was photographed using a cell imager. Migratory characteristic parameters such as confluence and gap fill in the scratch area were illustrated in Figure 4b, and migration rates were represented in Figure 4c.

PEITC Regulates Akt-mTOR and p38-STAT3 Pathways in SNU449 Cells

Protein expression levels were determined by semi-quantitative Western blotting analysis to investigate the impacts of the cisplatin and PEITC treatment on caspase 3, cleaved caspase 3, and CD44 protein levels in control, cisplatin- and PEITC-treated cells. Caspase 3 protein level was downregulated (0.47-fold change) in cisplatin-treated cells and upregulated (1.86-fold change) in PEITC-treated cells compared to control-treated cells. Cleaved caspase 3 protein level was upregulated in both cisplatin-treated cells (6.89-fold change) and PEITC-treated cells (1.09-fold change) compared to control-treated cells. CD44 protein level was downregulated in cisplatin-treated cells (0.54-fold change) and upregulated in PEITC-treated cells (3.2-fold change) compared to control-treated cells (Figure 5a and 5b).

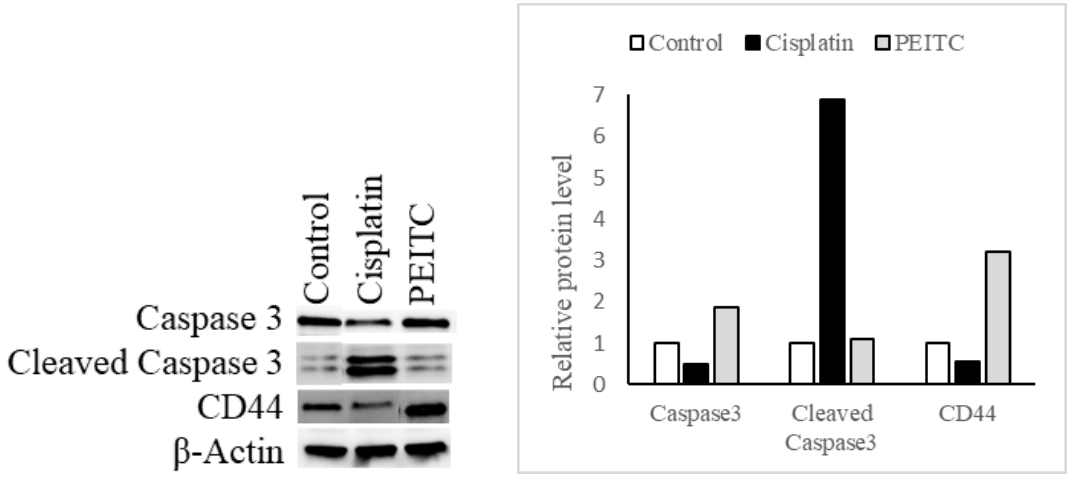

Figure 5. a. Protein expression changes of caspase 3 and CD44 determined by Western blotting in SNU449 cells following cisplatin and PEITC treatment for 24 h. **b.** Relative protein expression levels.

Furthermore, Akt-mTOR and p38-STAT3 signaling pathways proteins were investigated in control, cisplatin- and PEITC-treated cells. In cisplatin-treated cells compared to corresponding control-treated cells, protein levels of Akt, p-Akt, mTOR, p-mTOR, STAT3, and p-STAT3 were downregulated, while p38 and p-p38 were upregulated (Figure 6a). Fold changes were 0.75, 0.68, 0.75, 0.86, 0.59, and 0.0 for Akt, p-Akt, mTOR, p-mTOR, STAT3, and p-STAT3, respectively, and 1.69 and 1.24 for p38 and p-p38 (Figure 6b). In PEITC-treated cells compared to corresponding control-treated cells, protein levels of p-Akt, mTOR, p-mTOR, p38, p-p38, and p-STAT3 were upregulated, while Akt and STAT3 were downregulated (Figure 6a). Fold changes were 1.21, 1.42, 1.39, 1.69, 2.25, and 1.66 for p-Akt, mTOR, p-mTOR, p38, p-p38, and p-STAT3, respectively, and 0.83 and 0.68 for Akt and STAT3 (Figure 6b). Also, p-Akt/Akt, p-mTOR/mTOR, p-p38/p38, and p-STAT3/STAT3 ratios were calculated. p-Akt/Akt ratio was 0.9, p-mTOR/mTOR ratio was 1.15, p-p38/p38 ratio was 0.73, and p-STAT3/STAT3 ratio was 0.0 in cisplatin-treated cells compared to the corresponding control-treated cells. p-Akt/Akt ratio was 1.45, p-mTOR/mTOR ratio was 0.98, p-p38/p38 ratio was 1.33, and p-STAT3/STAT3 ratio was 2.41 in PEITC-treated cells when compared to the null treated control group (Figure 6c).

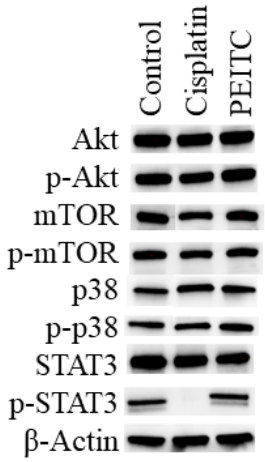

Figure 6a.

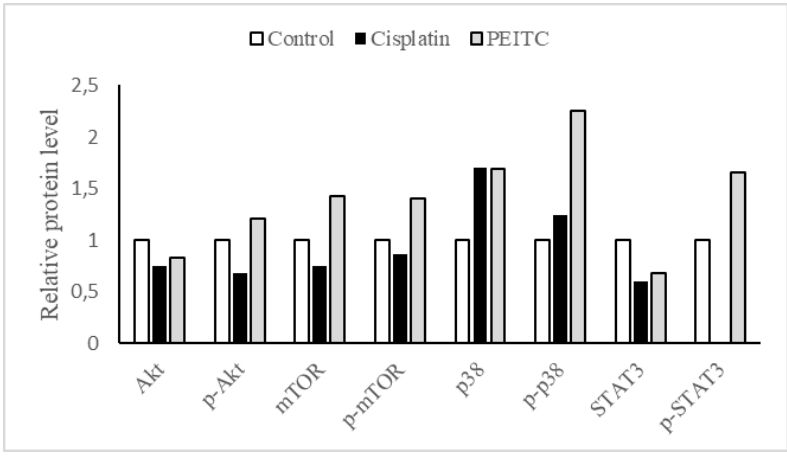

Figure 6b.

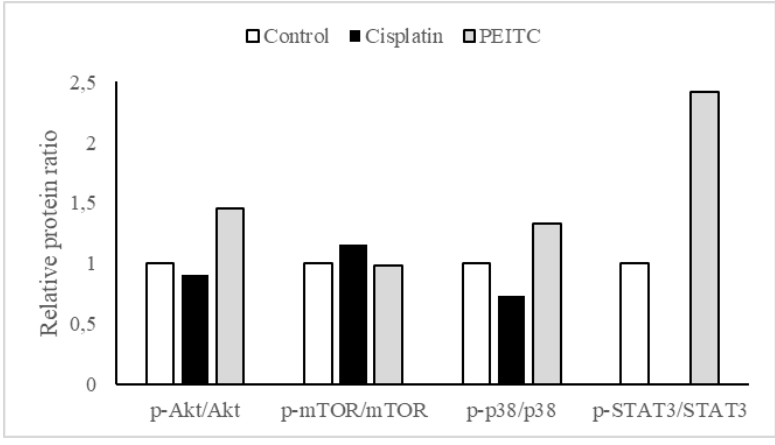

Figure 6c.

Figure 6. a. Protein expression changes of Akt/mTOR and p38/STAT3 pathways determined by Western blotting in SNU449 cells following cisplatin and PEITC treatment for 24 h. **b.** Relative protein expression levels **c.** The ratios of the phosphorylated state to the native state of Akt, mTOR, p38, and STAT3.

In the present study, the effect of PEITC on the cancer stem cell characteristic of the SNU449 HCC cell line was investigated. As a cancer stem cell marker, CD44 protein expression level was increased, and Akt-mTOR and p38-STAT3 signaling pathways were upregulated with PEITC treatment. Also, PEITC has promoted the migratory and proliferative properties of SNU449 cells. Based on results, PEITC modulated cancer stem cell phenotype by increasing CD44 expression through mainly the STAT3 pathway.

Evidence reveals that CSCs lead to treatment resistance, tumor progression, recurrence, and metastasis by affecting crosstalk between CSCs and the tumor microenvironment (Lv, Chen, Du, Zhou, & Tang, 2021). CD44 is not only one of the well-known cancer stem cell markers for HCC, but also for gastric, colorectal, and breast cancers (Koyama et al., 2020). In a study supported with clinical samples, CD44 expression levels have been reported to be positively correlated with the invasiveness degree of HCC cells, and this has been attributed to the metastasis-promotive effect of CD44. Also, CD44 correlates with tumor size, AFP level, and overall survival (P. Xie et al., 2022). In this study, following PEITC treatment, a roughly three-fold increase in the CD44 protein expression levels was measured. In line with the CD44 protein upregulation, PEITC affected the migration and proliferation characteristics of SNU449 cells. As an indicator of proliferation, the percentage of gap fill increased, while half-gap time decreased. Moreover, PEITC caused an increment in the overall migration rate of SNU449 cells. The cleaved caspase 3 to caspase 3 ratio, an apoptotic index, remained unchanged with PEITC treatment, while the ratio reached 15 in response to cisplatin exposure. These data suggest that PEITC promoted proliferative and migratory characteristics of SNU449 cells through CD44 upregulation. The novelty of the present study comes from the fact that it is the first study to show the CSC-promoting effects of PEITC in HCC cell line SNU449.

In addition to self-renewal capacity and differentiation potential, cancer and normal stem cells both have some essential regulatory pathways in proliferation, differentiation, survival, and self-renewal and immune modulation through the modification of specific pathways such as Wingless/Int-1 (Wnt)/β-catenin, Notch, nuclear factor kappa B (NF-κB), transforming growth factor beta/small mother against decapentaplegic (TGF-β/SMAD) PI3K/Akt/mTOR and JAK/STAT (Borlongan & Wang, 2023; Karami fath et al., 2022). PI3K/Akt/mTOR pathway is involved in different cellular processes of tumorigenesis, including cancer initiation, growth and proliferation, and migration and metastasis. Inhibition of the PI3K/Akt/mTOR pathway decreases stemness characteristics of CSCs by suppressing the expression of cancer stem cell markers, including CD44 in ovarian cancer and, dual inhibition of the pathway prevents the proliferation of CSCs in colon cancer (Chen et al., 2015; Deng et al., 2019). In HCC, using an mTOR inhibitor has reduced CD44 protein expression in SNU423 and SNU449 cell lines (Kim, Jiang, Badawi, & Schmittgen, 2017). In the presented study, the activated form of the members of the Akt/mTOR signaling pathway, p-Akt and p-mTOR were found to be increased in PEITC-treated cells. Despite the rising levels of the phosphorylated form of Akt and mTOR, only p-Akt/Akt ratio was elevated. Due to the accompanying high expression level in mTOR and p-mTOR, p-mTOR/mTOR ratio was found unchanged. Therefore, the regulatory effect of the Akt/mTOR signaling pathway on CD44 expression in PEITC-treated cells could be seen at a limited level.

STAT3, a member of JAK/STAT signaling, is responsible for the immune modulation capability of CSCs and the formation of a suitable microenvironment for tumor progression (Owen, Brockwell, & Parker, 2019)including those that are involved in tumor cell recognition and tumor-driven immune escape. Antitumor immune responses are largely driven by STAT1 and STAT2 induction of type I and II interferons (IFNs). The continuous active state of STAT3 causes cell survival and maintenance of stemness of breast cancer cells (L. Yang et al., 2020). Tumor-as-

sociated macrophages have promoted the expansion of CD44-positive CSCs by activating STAT3 signaling in HCC cells (Wan et al., 2014). Contrary to the observation in the Akt/mTOR signaling pathway, phosphorylated forms of p38 and STAT3 but also p-p38/p38 and chiefly p-STAT3/STAT3 ratios were observed as increased in PEITC-treated cells. Consistent with the previous reports, our results imply that CD44 upregulation was strongly driven by STAT3.

CONCLUSION

The results of this study unveil that STAT3-mediated upregulation of CD44 expression leads to the gain of cancer stem cell phenotype of PEITC-treated SNU449 cells. To elicit the exact contribution of mTOR and STAT3 pathways on the regulation of CD44 expression, a more comprehensive analysis with specific inhibitors of mTOR and STAT3 was needed. To the best of our knowledge, this is the first paper clarifies that PEITC regulates the cancer stem cell phenotype of SNU449 HCC cells via the STAT3-CD44 axis.

CONFLICT OF INTEREST

The author declares that there is no conflict of interest.

REFERENCES

Askan, G., Sahin, I. H., Chou, J. F., Yavas, A., Capanu, M., Iacobuzio-Donahue, C. A., Basturk, O., O'Reilly, E. M. (2021). Pancreatic cancer stem cells may define tumor stroma characteristics and recurrence patterns in pancreatic ductal adenocarcinoma. *BMC Cancer*, *21*, 385. https://doi.org/10.1186/s12885-021-08123-w

Ayob, A. Z., & Ramasamy, T. S. (2018). Cancer stem cells as key drivers of tumour progression. *Journal of Biomedical Science*, *25*, 20. https://doi.org/10.1186/s12929-018-0426-4

Borlongan, M. C., & Wang, H. (2023). Profiling and targeting cancer stem cell signaling pathways for cancer therapeutics. *Frontiers in Cell and Developmental Biology*, *11*(May), 1–33. https://doi.org/10.3389/fcell.2023.1125174

Chen, J., Shao, R., Li, F., Monteiro, M., Liu, J. P., Xu, Z. P., & Gu, W. (2015). PI3K/Akt/mTOR pathway dual inhibitor BEZ235 suppresses the stemness of colon cancer stem cells. *Clinical and Experimental Pharmacology and Physiology*, *42*(12), 1317–1326. https://doi.org/10.1111/1440-1681.12493

Deng, J., Bai, X., Feng, X., Ni, J., Beretov, J., Graham, P., & Li, Y. (2019). Inhibition of PI3K/Akt/mTOR signaling pathway alleviates ovarian cancer chemoresistance through reversing epithelial-mesenchymal transition and decreasing cancer stem cell marker expression. *BMC Cancer*, *19*(1), 618. https://doi.org/10.1186/s12885-019-5824-9

Karami fath, M., Ebrahimi, M., Nourbakhsh, E., Hazara, A. Z., Mirzaei, A., Shafieyari, S., Salehi, A., Hoseinzadeh, M., Payandeh, Z., Barati, G. (2022). PI3K/Akt/mTOR signaling pathway in cancer stem cells. *Pathology - Research and Practice*, *237*, 154010. https://doi.org/10.1016/j.prp.2022.154010

Kim, J., Jiang, J., Badawi, M., & Schmittgen, T. D. (2017). miR-221 regulates CD44 in hepatocellular carcinoma through the PI3K-AKT-mTOR pathway. *Biochemical and Biophysical Research Communications*, *487*(3), 709–715. https://doi.org/10.1016/j.bbrc.2017.04.121

Koyama, S., Tsuchiya, H., Amisaki, M., Sakaguchi, H., Honjo, S., Fujiwara, Y., & Shiota, G. (2020). NEAT1 is Required for the Expression of the Liver Cancer Stem Cell Marker CD44. *International Journal of Molecular Sciences*, *21*(6), 1927. https://doi.org/10.3390/ijms21061927

Lv, D., Chen, L., Du, L., Zhou, L., & Tang, H. (2021). Emerging Regulatory Mechanisms Involved in Liver Cancer Stem Cell Properties in Hepatocellular Carcinoma. *Frontiers in Cell and Developmental Biology*, *9*(July), 1–13. https://doi.org/10.3389/fcell.2021.691410

Owen, K. L., Brockwell, N. K., & Parker, B. S. (2019). Immune Regulation and Cancer Progression. *Cancers*, *11*(12), 2002. https://doi.org/10.3390/cancers11122002

Sayiner, M., Golabi, P., & Younossi, Z. M. (2019). Disease Burden of Hepatocellular Carcinoma : A Global Perspective. *Digestive Diseases and Sciences*, *64*(4), 910–917. https://doi.org/10.1007/s10620-019-05537-2

Shin, J. M., Lim, E., Cho, Y. S., & Nho, C. W. (2021). Cancer-preventive effect of phenethyl isothiocyanate through tumor microenvironment regulation in a colorectal cancer stem cell xenograft model. *Phytomedicine*, *84*, 153493. https://doi.org/10.1016/j.phymed.2021.153493

Song, L., Zhang, H., Hu, M., Liu, C., Zhao, Y., Zhang, S., & Liu, D. (2021). Sinomenine inhibits hypoxia induced breast cancer side population cells metastasis by PI3K / Akt / mTOR pathway. *Bioorganic & Medicinal Chemistry*, *31*, 115986. https://doi.org/10.1016/j.bmc.2020.115986

Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A., & Bray, F. (2021). Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA: A Cancer Journal for Clinicians*, *71*, 209–249. https://doi.org/10.3322/caac.21660

Wan, S., Zhao, E., Kryczek, I., Vatan, L., Sadovskaya, A., Ludema, G., Simeone, D. M., Zou, W., Welling, T. H. (2014). Tumor-associated macrophages produce interleukin 6 and signal via STAT3 to promote expansion of human hepatocellular carcinoma stem cells. *Gastroenterology*, *147*(6), 1393–1404. https://doi.org/10.1053/j.gastro.2014.08.039

Xie, P., Yan, J., Wu, M., Li, H., Chen, Z., Yu, M., Zhang, B., Chen, L., Jin, L., Zhou, B., Li, X., Xiao, Y., Xu, Y., Long, J., Zhang, J., Guo, L. (2022). CD44 potentiates hepatocellular carcinoma migration and extrahepatic metastases via the AKT/ERK signaling CXCR4 axis. *Annals of Translational Medicine*, *10*(12), 1–16. https://doi.org/10.21037/atm-22-2482

Xie, X. P., Laks, D. R., Sun, D., Ganbold, M., Wang, Z., Pedraza, A. M., Bale, T., Tabar, V., Brennan, C., Zhou, X., Parada, L. F. (2022). Quiescent human glioblastoma cancer stem cells drive tumor initiation , expansion , and recurrence following chemotherapy. *Developmental Cell*, *57*(January 10), 32–46. https://doi.org/10.1016/j.devcel.2021.12.007

Yang, B., Li, M., Tang, W., Liu, W., Zhang, S., Chen, L., & Xia, J. (2018). Dynamic network biomarker indicates pulmonary metastasis at the tipping point of hepatocellular carcinoma. *Nature Communications*, *9*, 678. https://doi.org/10.1038/s41467-018-03024-2

Yang, L., Shi, P., Zhao, G., Xu, J., Peng, W., Zhang, J., Zhang, G., Wang, X., Dong, Z., Chen, F., Cui, H. (2020). Targeting cancer stem cell pathways for cancer therapy. In *Signal Transduction and Targeted Therapy*, *5*, 8. https://doi.org/10.1038/s41392-020-0110-5

Yao, H., Sun, L., Li, J., Zhou, X., Li, R., Shao, R., Zhang, Y. Li, L. (2020). A Novel Therapeutic siRNA Nanoparticle Designed A Novel Therapeutic siRNA Nanoparticle Designed for Dual-Targeting CD44 and Gli1 of Gastric Cancer Stem Cells. *International Journal of Nanomedicine*, *15*, 7013–7034. https://doi.org/10.2147/IJN.S260163

Yun, J. H., Kim, K. A., Yoo, G., Kim, S. Y., Shin, J. M., Kim, J. H., Jung, S. H., Kim, J., Nho, C. W. (2017). Phenethyl isothiocyanate suppresses cancer stem cell properties in vitro and in a xenograft model. *Phytomedicine*, *30*, 42–49. https://doi.org/10.1016/j.phymed.2017.01.015

Zhang, H.-L., Wang, P., Lu, M.-Z., Zhang, S.-D., & Zheng, L. (2019). c - Myc maintains the self - renewal and chemoresistance properties of colon cancer stem cells. *Oncology Letters*, *17*, 4487–4493. https://doi.org/10.3892/ol.2019.10081