Research Paper

Investigation of NSD2 Protein Expression Changes in Hepatocellular Carcinoma Cells After Treatment With Curcumin and Phthalates

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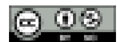

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ABSTRACT

Background: Hepatocellular carcinoma (HCC) is one of the most common types of cancer worldwide. The role of molecular markers in the development and progression of this cancer has been extensively studied. The overexpression of the 90-kilobase protein methyltransferase NSD2 (nuclear receptor binding SET domain- protein 2) is associated with tumor development and some types of cancers. This study aims to investigate the changes in NSD2 protein expression in HCC cells after treatment with curcumin and phthalates.

Method: This study compared the NSD2 protein expression in HepG2 cancer cells and fibroblast cells that were either untreated or treated with the half-maximal inhibitory concentration (IC_{50}%) of phthalates, curcumin, or their combination. The Western blot method and protein quantification were used to detect and determine NSD2 protein levels, and ImageJ software was used to analyze the desired bands.

Results: Curcumin, phthalates, and their combination reduced the expression of NSD2 in HepG2 cancer cells and normal fibroblast cells compared to untreated cells ($P<0.001$). This decrease in expression was more significant in cells treated with both curcumin and phthalates than in treatment with curcumin or phthalates alone.

Conclusion: The IC_{50}% of curcumin, phthalates, and their combination can reduce *NSD2* expression, where the effect of the combined form is greater. Therefore, the combination of phthalates and curcumin is recommended as a potential anti-cancer agent against HCC cells with an effect on reducing NSD2 expression.

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1. Introduction

Liver cafncer is the fifth most common cancer and the fourth leading cause of cancer-related death worldwide. Hepatocellular carcinoma (HCC) is the most common primary malignant liver tumor. Studies have shown that distinct morphological phenotypes of HCC are associated with different genetic defects and biological pathways that contribute to tumor growth [1]. Genetic and morphological pathways can help identify the mechanisms of cancer progression and carcinogenesis, predict/diagnose diseases, and develop treatment methods [2]. The identification of genes involved in cancer development has recently received significant attention from researchers worldwide due to the important role of cancer pathophysiology at the cellular and molecular levels in managing and monitoring cancer [3]. Molecular markers for diagnosing and evaluating treatment methods are highly important. Therefore, developing strategies for early detection, prediction, prognosis, and treatment of patients with HCC is essential [4].

One of the molecular markers with a role in cancer development is the *NSD2* (nuclear receptor binding SET domain-protein 2). The 90-kilobase protein methyltransferase *NSD2* belongs to the NSD protein lysine methyltransferases (KMT) family that can change the methylation status. Studies have shown that the overexpression of *NSD2* is associated with tumor development and some types of cancers. In multiple myeloma, overexpression of *NSD2* is due to fusions to IgH via t (4;14) translocations or recurrent E1099K mutations, leading to increased methyltransferase activity in lymphoma [5, 6]. Overexpression of *NSD2* has also been reported in some cancers, such as endometrial cancer, head and neck squamous cell carcinoma, neuroblastoma, lung cancer, ovarian cancer, bladder cancer, colorectal cancer, prostate cancer, and HCC [7, 8]. Studies have shown that *NSD2* supports the proliferation of cancer cell lines such as myeloma cell lines, E1099K-carrying leukemic cell lines, fibrosarcoma, and others [9-11]. *NSD2* plays a role in many cellular processes, most of which may contribute to tumor formation. However, the effect of *NSD2* expression on tumor formation is still unclear [12]. Studies have shown that *NSD2* is overexpressed in HCC and is associated with the Edmondson stage and vascular invasion. *NSD2* is also overexpressed in solid tumors; such abnormal expression is associated with the occurrence and development of cancer. A significant reduction in *NSD2* inhibits cell proliferation, migration, invasion, and the epithelial-mesenchymal transition (EMT) process, while overexpression of *NSD2* has the opposite effect and acts as an oncogene in solid tumors.

The role of *NSD2* in repair of human DNA damage is evident, and recent studies have supported the role of *NSD2* in tumor invasion and metastasis through the EMT process [13]. *NSD2* regulates the EMT-associated proteins, and reduced *NSD2* increases the level of E-cadherin protein expression and N-cadherin and vimentin protein levels. *NSD2* increases the proliferation of cancer cells by regulating cancer targets such as tumor necrosis factor α (*TNF-α*), interleukin-6 (*IL-6*), and interleukin-8 (*IL-8*), or by reducing p53 stabilization and also plays a role in regulating the cell cycle and facilitating natural DNA replication [14, 15]. Overall, given the potential role of *NSD2* in HCC, this study aims to investigate the expression of *NSD2* and its mechanism of action in HCC by applying two chemical substances with anti-cancer (curcumin) and carcinogenic (phthalates) properties on the HepG2 HCC cell line.

2. Materials and Methods

Cell culture

The HepG2 cell line, which represents HCC, as well as the fibroblast cell line, which represents normal cells, were purchased from the Iranian Genetic Resources Center. Both cell lines were cultured according to the protocol provided by American Type Culture Collection (ATCC) [16]. The cells were incubated at 37°C, 95% humidity, and 5% CO_2. Once the cell density reached 90%, the cells were passaged using Trypsin-EDTA solution 0.05%. The Dulbecco's Modified Eagle Medium was used for both cell lines based on the ATCC protocol, with 10% fetal bovine serum and 1% Pen-strep added to the medium [17].

Determination of cell viability

To determine the percentage of viable cells, 100 µL of a uniform cell suspension was pipetted into a sterile test tube, and an equal volume of Trypan blue was added. After a few minutes of incubation and pipetting, one drop of the mixture was taken, and the cells were counted using a hemocytometer under a microscope. The percentage of viable cells was calculated using Equation 1:

1. *100×(total number of dead and live cells)/Number of live cells=Percentage of viable cells*

Assessment of cytotoxicity effects

To investigate the cytotoxic effects of drug compounds on the growth and proliferation of cancer cells and determine the half-maximal inhibitory concentration (IC_{50})

of these compounds, the MTT colorimetric assay was used [18]. Phthalate and curcumin drugs were purchased from Sigma Aldrich Company, and both normal and cancer cells were treated with different concentrations of Phthalate (15, 20, 25, and 30 µg/mL), Curcumin (10, 20, 30, 40, 50, and 60 µg/mL), and their combination (15, 20, 25, and 30 µg/mL) for 24, 48, and 72 hours. Then, the culture medium was removed and replaced with 100 microliters of the prepared concentrations. The percentage of cell viability was calculated using Equation 2:

2. *% Cell viability=(Mean absorbance of treated cells/Mean absorbance of control cells)×100*

To determine the 50% lethal dose of drugs on the cancer cell line, the obtained toxicity information (percentage of toxicity) from the treated samples and controls (untreated cells) were analyzed in the Pharm-PCS system [19], and the corresponding IC_{50} values were accurately determined.

Determination of *NSD2* level

The NSD2 was detected using the IHCPlus™ Polyclonal Rabbit anti-Human WHSC1/NSD2 Antibody (catalog number LS-B8065) and the Western Blot method. First, a piece of polyvinylidene difluoride (PVDF) paper was cut to the size of the gel and placed in pure methanol for one minute to prepare the blotting paper. Then, the paper was removed from methanol and placed in the transfer buffer (0.14 g glycine and 0.33 g Tris base dissolved in 500 mL of deionized water with 200 mL of methanol). The desired piece of polyacrylamide gel was cut and placed in the transfer buffer for 15 minutes to equilibrate with it. The transfer sandwich (the gel sandwiched between 8 filter papers and the PVDF paper) was used to transfer the gel, placed in the transfer tank containing the transfer buffer.

Since the primary and secondary antibodies in Western Blot have a high binding capacity, non-specific sites were blocked using a blocking solution including 5% non-fat dry milk in tris-buffered saline/Tween (TBST). Finally, the PVDF paper was washed once for one minute with a TBST solution, and the primary monoclonal antibody against the NSD2 enzyme (at a dilution of 1:500) from LSBio Company and the primary monoclonal antibody against β-Actin (at a dilution of 1:800) from Santa Cruz Company (catalog number sc4777) were diluted in TBST solution containing 5% bovine serum albumin. Then, the membrane was incubated over night (for at least 15 hours) with the primary antibody solution on a shaker in a refrigerator at 4˚C.

To confirm the expression of NSD2 protein in transfected cells, cellular proteins were electrophoresed in the presence of odium dodecyl sulfate (SDS) and stained with Coomassie blue on a polyacrylamide gel. The transfected cells were then collected, and crude cellular protein was prepared. Subsequently, the cell lysates were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a membrane using monoclonal anti-NSD2 antibodies to confirm the production of the desired protein.

To quantify the NSD2 protein, the desired bands were identified using Image-J software. The software calculated the results quantitatively based on pixel density. The obtained value for NSD2 protein was normalized to the internal control of each tissue, and the data were entered into the SPSS software for analysis.

Statistical analysis

The statistical analysis of the collected data was performed using the chi-square test, Fisher's exact test, independent t-test, and one-way ANOVA in SPSS software, version 22.

3. Results

Gene expression changes

According to the results illustrated in Figure 1, the expression of NSD2 protein was significantly reduced in HepG2 cancer cells and non-cancerous cells treated with curcumin (30 µM) compared to the untreated cells ($P<0.001$). However, this decrease in protein expression was not significant in fibroblast cells treated with curcumin compared to untreated fibroblast cells ($P>0.05$).

According to the results illustrated in Figure 2, the expression of NSD2 protein was significantly reduced in cancer and non-cancerous cells treated with phthalates (25 µM) compared to untreated cells ($P<0.05$). However, this decrease in protein expression was not significant in fibroblast cells treated with phthalates compared to untreated fibroblast cells ($P>0.05$).

The results of studying the changes in NSD2 protein expression in HepG2 cancer cells and normal fibroblast cells treated with IC_{50} of curcumin+phthalates mixture (30 µM of each drug) showed a significant reduction in NSD2 protein expression in cancer and non-cancerous cells under treatment compared to untreated cells ($P<0.001$). However, this decrease in protein expression was not significant in fibroblast cells treated with both

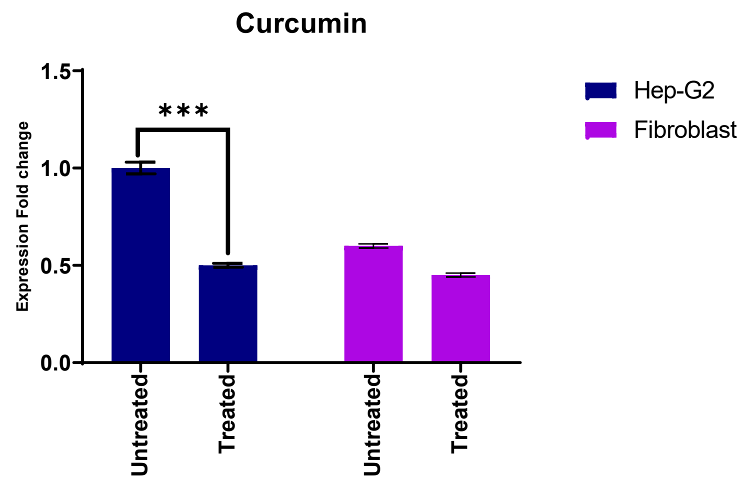

Figure 1. NSD2 protein expression changes in hepG2 and fibroblast cells treated with IC_{50} of curcumin with three repetitions
***P<0.001

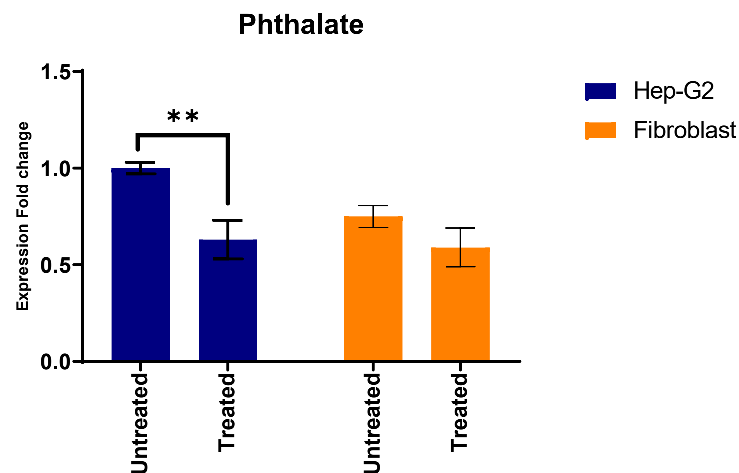

Figure 2. NSD2 protein expression changes in hepG2 and fibroblast cells treated with IC_{50} of phthalates with three repetitions
**P<0.05

curcumin and phthalate compared to untreated fibroblast cells (P>0.005) (Figure 3). Obtained results showed that this reduction in expression in cells treated with the mix of two drugs was greater than in cells treated with a single drug (Figure 4). The results of SDS-PAGE and Western blotting confirmed that the *NSD2* expression was reduced when treated with IC_{50} of two drugs (Figure 5).

4. Discussion

The findings of the present study showed that low concentrations of phthalates could promote cell growth and proliferation, but the increase of concentrations led to the death of cancer and non-cancerous cells. In addition, the combination of curcumin and phthalate at high concentrations significantly reduced the survival of cancer cells. The IC_{50} of curcumin, phthalates, and their mixture caused a decrease in NSD2 protein expression, where the effect of combined form was significantly greater. Therefore, the combination of phthalates and curcumin can be considered an anti-cancer agent against HCC cells by reducing NSD2 protein expression.

Studies have shown that treatment with curcumin inhibits the growth of liver cancer stem cells, induces cellular apoptosis, and regulates the expression of proteins related to apoptosis and the release of cytochrome C. In addition, curcumin treatment prevents the activa-

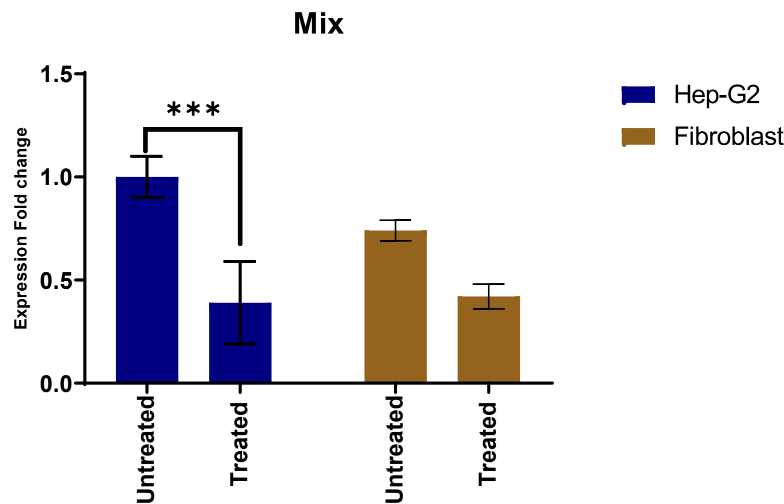

Figure 3. NSD2 protein expression changes in hepG2 and fibroblast cells treated with IC_{50} of curcumin+phthalate with three repetitions

***P<0.001

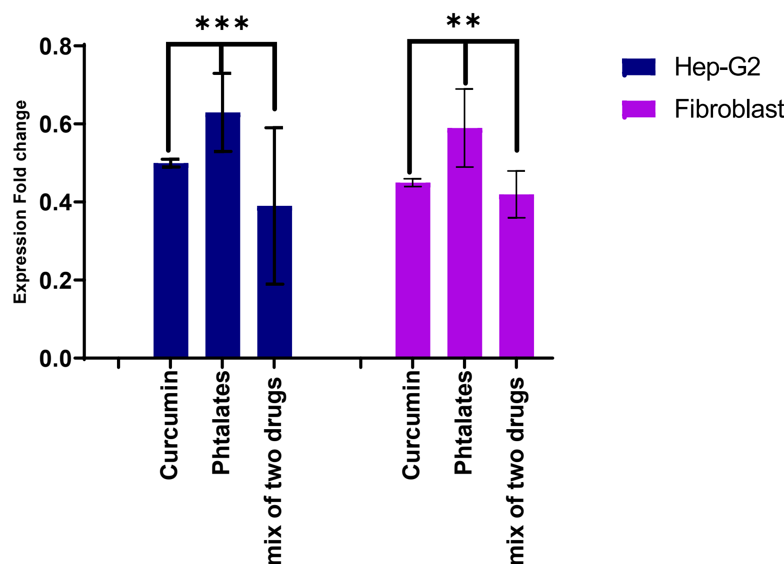

Figure 4. NSD2 protein expression changes in hepG2 and fibroblast cells treated with curcumin, phthalates, and their combination

***P<0.001, **P<0.05

tion of the phosphatidylinositol 3-kinase/protein kinase B signaling pathway. Moreover, curcumin has been suggested as an effective agent in treatment of liver cancer [20]. Phthalates have a role in the migration of HCC cells by increasing the interaction between the pregnane X receptor (PXR) and a specific E26 transformation sequence (ETS-1). Phthalates activate the transcription of ETS-1 and PXR, whereby the activated PXR can directly bind to and enhance the activity of ETS-1, leading to the induction of ETS-1 target genes associated with invasion in HCC [21]. Other evidence has shown that phthalates promote cancer growth, including cell proliferation, migration, and invasion. Furthermore, phthalates promote the development of EMT in HCC cells. However, curcumin can suppress phthalate-induced cell migration, invasion, and EMT, reduce the proportion of pseudo-cancer stem cells in liver cancer cell lines under laboratory conditions, and inhibit tumor growth and metastasis in the body [22].

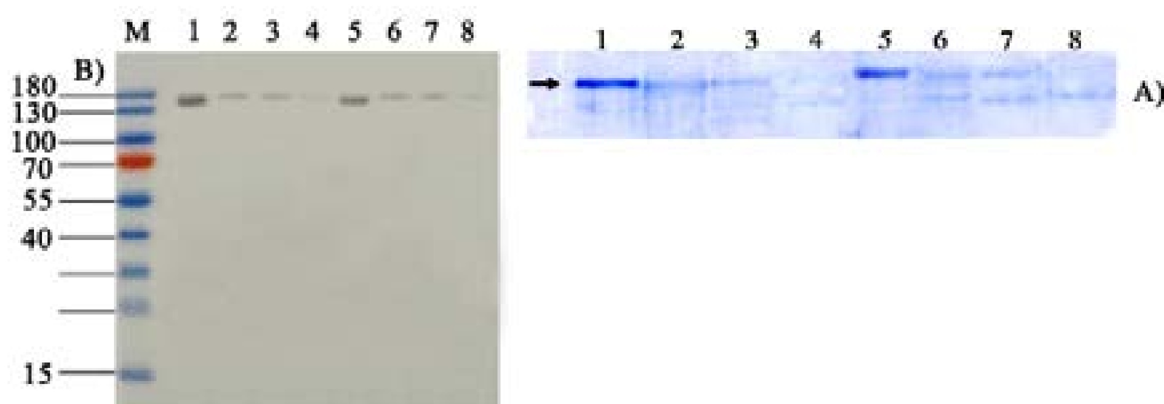

Figure 5. Results of SDS-PAGE and Western blotting for the *NSD2* gene in two cell lines, fibroblast and HepG2

A) SDS-PAGE results showing the lysates of transfected cells; 1-4 in fibroblast cells and 5-8 in HepG2 cancer cells. Lanes 1 & 5: Positive controls; Lanes 2 & 6: Cells under treatment with the IC_{50} of curcumin; Lanes 3 & 7: Cells under treatment with IC_{50} of phthalates; Lanes 4 & 8: Cells under treatment with IC_{50} concentration of both drugs.

B) Western blot results using monoclonal anti-NSD2 antibodies for detecting the NSD2 protein, 1-4 in fibroblast cells, 5-8 in HepG2 cancer cells

Lanes 1 & 5: Positive controls; Lanes 2 & 6: Cells under treatment with IC_{50} of curcumin; Lanes 3 & 7: Cells under treatment with IC_{50} of phthalates; lanes 4 & 8: Cells under treatment with IC_{50} of both drugs, lane M: Protein marker.

NSD2 is a histone methyltransferase that catalyzes H3 histone dimethylation at lysine 36, and it a critical molecule in proliferation, metastasis, and tumorigenesis. However, its role in tumor angiogenesis is still unknown. A recent study showed that *NSD2* promoted tumor angiogenesis under in-vitro and in-vivo conditions. Moreover, the study confirmed that the angiogenic function of *NSD2* is mediated by signal transducer and activator of transcription 3 (*STAT3*). The authors foud that *NSD2* promotes methylation and activation of *STAT3*. Moreover, mass spectrometry and mutagenesis assays showed that *NSD2* methylated *STAT3* at lysine 163 (K163). Meanwhile, K to R mutant at K163 of *STAT3* reduced the activation and angiogenic function of *STAT3*. The authors concluded that the methylation of *STAT3* catalyzed by *NSD2* ehnaces the activation of *STAT3* pathway and promotes tumor angiogenic ability [23]. Previous studies have shown that enhancer of zeste homolog 2 (EZH2), which catalyzes H3K27 methylation and is related to gene silencing, is an important oncogenic stimulus and may have potential therapeutic roles in human cancers. NSD2 mediates the Wnt pathway, NF-kB signaling for oncogenesis, proliferation, and survival [24].

García-Carpizo et al. showed that the NSD2 protein is involved in lung cancer [25]. Han et al. demonstrated that NSD2 plays a role in the progression of kidney cancer [26]. In a study by Stangl-Kremser et al., NSD2 expression was found to have a role in advanced prostate cancer [27]. In a study by Zhao et al., NSD2 was found to play an important oncogenic role in colorectal cancer [12]. Based on the results of conducted studies, the increase in NSD2 protein expression is associated with the incidence, prognosis, and progression of cancer. Therefore, reducing the expression of this protein can play a crucial role in reducing the incidence and improving the prognosis of HCC. The use of curcumin and phthalates can attenutae the expression of *NSD2* and reduce the incidence, improve the prognosis, and prevent the progression of HCC.

5. Conclusion

Curcumin is as an effective anti-cancer agent for treating HCC. Low concentrations of phthalates can promote cell growth and proliferation. However, the increase of its concentration can lead to the death of both cancer and non-cancerous cells and induction of apoptosis. Using the combination of curcumin and phthalates at high concentrations can result in reduction of cancer cell viability. Furthermore, the IC_{50} of curcumin, phthalates, and their combination can reduce the expression of the NSD2 protein, where the effect of combined form is significantly greater. Therefore, we suggest the mixture of phthalates and curcumin as a promising anti-cancer agent against HCC cell lines, with the potential to reduce the expression of the NSD2 protein.

Ethical Considerations

Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

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Authors' contributions

All authors equally contributed to preparing this article.

Conflict of interest

The authors declared no conflict of interest.

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