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# ANTI-CANCER EFFECT OF CURCUMIN ON SURVIVAL AND EXPRESSION OF DNMT1 AND CDH1 GENES IN CELL LINE MIAPACA2

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**ABSTRACT**

**Introduction:** Pancreatic cancer is a deadly sinister cancer and the fourth leading reason for death worldwide. DNMT1 is essential for the conservation of the methylation landscape due to its ability to recognize hemimethylated DNA and conserve methylation during somatic cellular division.Ecad play a role in cellular connectivity through extracellular domains, Loss of Ecad Protein, lead to loss of Intercellular Connections, Provides Cell Metastasis. The pharmacological effects of curcumin include inducing apoptosis, anti-cell proliferation, antioxidant and anti-angiogenesis are proved and this compound has the potential to be used in cancer prevention.

**Objectives:** The current study was performed in order to explore in vitro antitumor activity of curcumin in human pancreatic carcinoma cell line MIAPaCa2.DNMT1 and CDH1 genes expression were examined by quantitative real-time PCR. Finally, the effects of curcumin on viability and DNMT1 gene and CDH1 gene expression status were evaluated.

Method: MiaPaca-2cell line was cultured in monolayers. The cells were treated with curcumin using different concentrations of 2,5, 10, 20, 40, 80 µM for 24, 48 and 72 hours. Viability was checked by MTT assay and DNMT1 and CDH genes expression was evaluated by RT-PCR.

Results: Our results indicate that the level of DNMT1 mRNA expression was decreased after treatment. Expression level of CDH1 mRNA were increased. Data obtained from MTT revealed antiproliferative effects of curcumin for 20,40,80 µM concentrations.

**Conclusions:** We conclude that cell viability and level of DNMT1 mRNA was decreased after curcumin treatment, and level of CDH1 mRNA was increased. So, These observations suggest that curcumin , a molecule with varied actions, as a supplementary could be developed into an effective chemopreventive and chemotherapeutic agent for pancreatic cancer treatment.

## 1.Introduction

Pancreatic cancer is a deadly sinister cancer and the fourth leading cause of death worldwide. Despite advances in recent years in the diagnosis and treatment of this cancer, The 5-year survival rate of these patients is below 5% and has a very poor prognosis (Zhang, Liang et al. 2011), (Yin, Wang et al. 2007). By 2030, it is anticipated to be the second murderous cancer in the world (Khan, Zubair et al. 2015). The only way to treat and save these patients is to have surgery and pancreatic resection, but since the disease is diagnosed late, only 10 to 20% of patients are candidates for surgery(Gillen, Schuster et al. 2010). At the time of diagnosis 15% of patients

are in stage 1 or 2, 35% of patients are in advanced stage of the disease and 50% of patients are diagnosed with stage 4 and metastasis(Suker, Beumer et al. 2016). So pancreatic surgery and resection do not help patients much.Their 5-year survival rate is 11% to 25% and only a few months longer will survive (Alexakis, Halloran et al. 2004). Studies on the molecular mechanism of pancreatic cancer display that this disease is related to genetic and epigenetic changes(Hong, Park et al. 2011). Epigenetics is a significant topic introduced as one of the pathways controlling gene expression, determined as the changes in gene expression without any essential changes in sequences(Azad, Kaviani et al. 2013, Rahmani, Azad et al. 2017). Methylation is one of the most substantial epigenetic mechanisms, It involves the addition of a methyl group to the cytosine base after DNA copying(Issa 2007). DNA methylation is carried out by DNA methyltransferase enzymes, It contains three enzymes DNMT1, DNMT3a and DNMT3b. DNMT1 is the significant enzyme for methylation after transcription(Baylin 2005). Epigenetic changes, including DNA methylation, alter gene expression and chromatin structure and changing DNA methylation patterns has a significant role in human cancer, these changes include increased methylation of the CpG islands that can inactivate the tumor suppressor(Kulis and Esteller 2010). Increased dnmt1 gene expression has been observed in most pancreatic cancer patients(Li, Omura et al. 2010).

Ecad glycoprotein encoded by CDH1 gene located on chromosome q22.116 and belong to the cell adhesion molecule (CAM) family. Intracellular and $Ca2^{+}$ dependent protein in epithelial cells and they play a role in cellular connectivity through extracellular domains and communication with next cell cadherins and operate as a tumor inhibitor (Huber, Züllig et al. 2011),(Vesuna, van Diest et al. 2008). Loss of Ecad Protein Function through CDH1 Gene Mutation, Loss of Intercellular Connections, Provides Cell Metastasis(Norton, Ham et al. 2007).

Curcumin is a phenolic compound that is the active compound of the plant Chromoma Longa, known as turmeric. Used as a spice and a compound known in traditional medicine in many countries(Gou, Men et al. 2011). It has anti-bacterial, anti-fungal, anti-yeast, anti-parasitic and anti-cancer effects(Anuchapreeda, Fukumori et al. 2012). The pharmacological effects of curcumin include apoptotic, anti-cell proliferation, antioxidant and anti-angiogenesis and this compound has the potential to be used in cancer prevention(Shehzad, Wahid et al. 2010). In addition to treating cancer as an anti-inflammatory, it is also used to treat Alzheimer's disease and malaria, It targets cell DNA, RNA and cell proteins (enzymes) (Yallapu, Jaggi et al. 2012). Curcumin inhibit multiple intracellular signaling pathways. Proteins of these pathways that target the chromatin include NF-κB, AP-1 COX-2, MMPs, EGFR, β-catenin and TNF. The anticancer effects of curcumin are related to targeting of COX-2, HER2, TNF, EGFR, Bcr-abl, proteasome PI3K, AKT, Ras and NF-κB proteins (Kasi, Tamilselvam et al. 2016).

The current study was performed in order to explore in vitro antitumor activity of curcumin against human pancreatic carcinoma cell line MIAPaCa2 and DNMT1 gene and CDH1 gene expression was assayed by quantitative real-time PCR. Finally, the effects of curcumin on viability and DNMT1 gene and CDH1 gene expression situation were evaluated.

## 2.Materials and methods

### 2.1.Cell culture

The MiaPaca-2 ،cell line was bought from Pasteur Institute of Iran and cultured in RPMI 1640 Gibco completed with 10% fetal bovine serum (FBS; Gibco) and 1% antibiotic (100 U/ml of penicillin, 10 mg/ml of streptomycin) at 37°C under an atmosphere of 5% CO2. The cells were cultured in cell culture plates, each well containing 6x105 cells for all concentration and control.

After treatment of the cells with curcumin using a concentration of 99% ethanol we prepared dilutions 2,5, 10, 20, 40 and 80 µM. At 24,48 and 72 hours, cells were harvested and isolated from medium.

Real-time reverse transcription-polymerase chain (real-time RT-PCR)

At a spacing of 24,48 and 72 hours, cells were harvested and isolated from medium.

using the kit iraizol RNA extraction (RNA biotechnology CO, Isfahan, Iran), RNA was extracted, according to the manufacturers. For the quantitative analysis of RNA extraction, we used Nanodrop techniques. For cDNA synthesis, we used the RBcDNA synthesis kit (RNA biotechnology CO, Isfahan, Iran). Real-time RT-PCR was carried out using the Rotor-Gene Q appliance (QIAGEN co.) and analyzed by Rest 2009 software.

Primers used for DNMT1 expression were designed by IDT and NCBI

(F) GTGGGGGACTGTGTCTCTGT

(R) TGAAAGCTGCATGTCCTCAC for forward and reverse respectively.

Primers used for CDH1 expression were designed by IDT and NCBI

(F) GGGTTAAGCACAACAGCAAC

(R) ACCTGACCCTTGTACGTGGT

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a RNA integrity control and amplified using primers

5′-CAATGACCCCTTCATTGACC-3′

5′-TGGAAGATGGTGATGGGATT-3′

for forward and reverserespectively. [22]

The cycles were as persued: 95°C for 5 minutes, followed by 40 cycles of denaturing at 95°C for 15 seconds, annealing at 60°C for 20 seconds and extension at 72°C for 20 seconds. This was followed by the final extension at 72°C for 10 minutes.

### 2.2.MTT assay

Cells were cultured in 96-well culture plate (2x103 cell/200µl). 24 hours after the culture, we replaced ambient with 0.1% ethanol as control and concentrations of 2,5, 10, 20, 40, and 80 µM curcumin. Each concentration was accomplished as a quadruplet. After 24, 48, 72 hours of treatment, cells were laundering with PBS and 50 µl MTT solution (Sigma) was added at a concentration of 0.05 mg/ml diluted in PBS Cells were incubated at 37°C for 4 hours to permit the formation of purple formazan crystals due to mitochondrial dehydrogenase activity. Then 200 µl of filtered DMSO was added to each well pursued by 25 µl glycine buffer. The absorbance of the cell suspension was measured by spectrophotometric at 570 nm using an ELISA reader ( BioTek ELX800, USA). Data were analysed by one-way analysis of variance ANOVA confirmed by Dunnett's multiple comparison test using Graphpad prism 8 software where $P< 0.05$ was considered to be statistically substantial.

## 3. Results and discussions

### 3.1.Evaluation of Cytotoxic Effect Using MTT Test

The MTT test using to estimate Metabolic acting that can be measuring the activity of a mitochondrial enzyme succinate dehydrogenase. MTT is planned for the description of a cytotoxic indicator in a cell population using a 96 well plate format.

In the present study, we applied the MTT test to evaluate the anticancer activity of Curcumin in cell line MIAPaCa-2 assay in both concentration and time-dependent manner as explained Material and methods section. As illustrated in Figure 1 the anchorage-dependent cell viability of MIAPaCa-2 cells after exposure to curcumin was decreased significantly. These data illustrate that curcumin has antiproliferative activity in pancreatic cancer cell lines.

### 3.2.Real-time PCR

### DNMT1 mRNA expression

Our results indicate that the level of expression DNMT1 mRNA, In 24 hours for 5, 10, 20,40 and 80 µM concentrations was decreased after treatment with curcumin, but not 2 µM. in 48 hours all of concentration was decreased and after 72 hours treatment with curcumin all of concentration was decreased (figure 2).

### 3.3.CDH1 mRNA expression

Level of expression CDH mRNA, in 24 hours for 40 and 80 µM concentration was increased, but not 5,10,20 µM. in 48 hours for

40 and 80 µM concentration was increased. after 72 hours all of concentration was increased (figure 3).

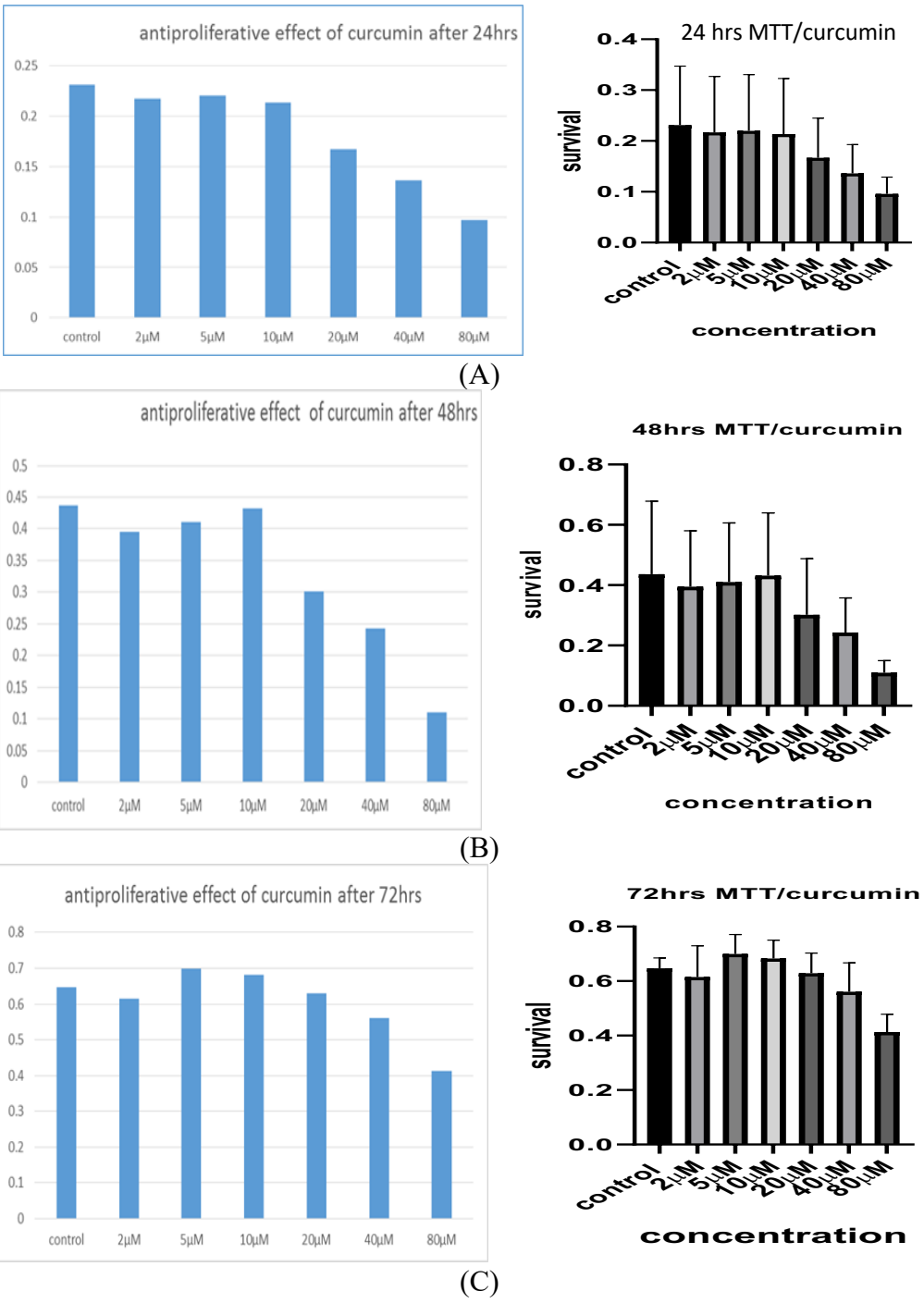
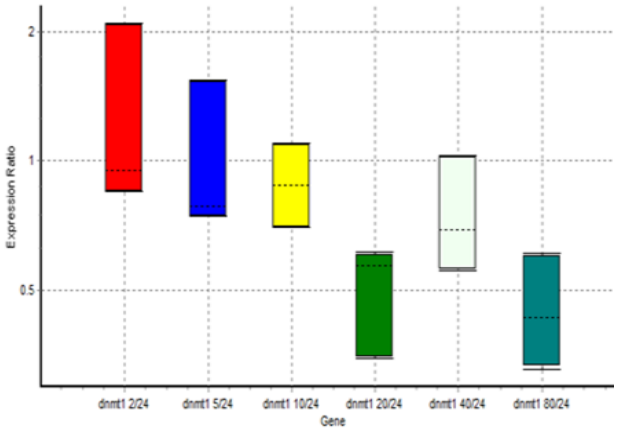
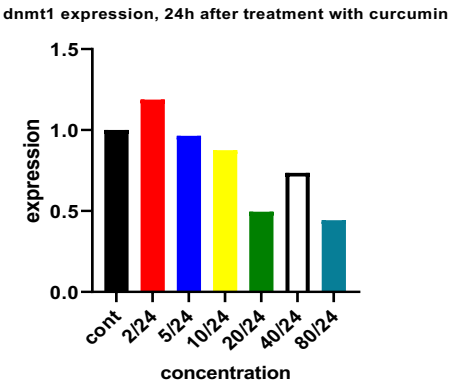
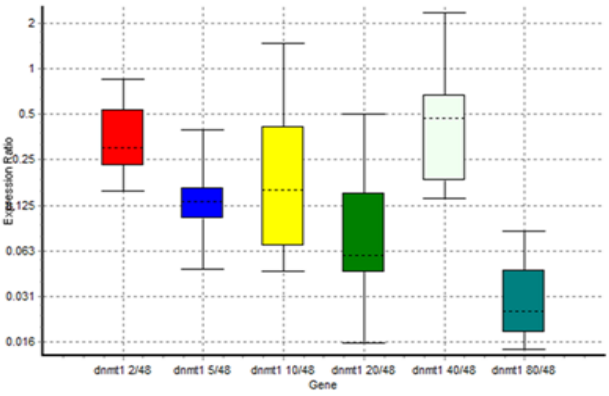


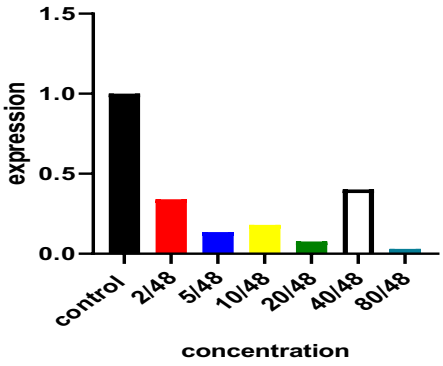

**Figure 1**. Anchorage-dependent cell viability of pancreatic cancer cell line (MiaPaca-2), (A) 24 hours after treatment with curcumin , (B) 48 hours after treatment with curcumin, (C) 72 hours after treatment with different concentrations (2,5,10,20,40,80µM) of curcumin. Antiproliferative effects were seen at 20 to 80 µM of curcumin with a time- and concentration-dependent manner ($P<0.05$).



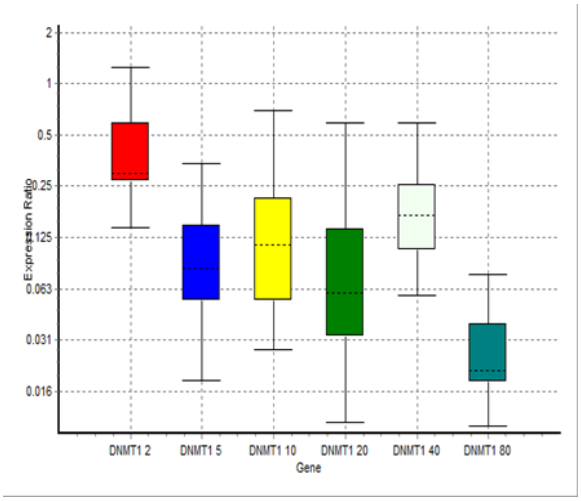



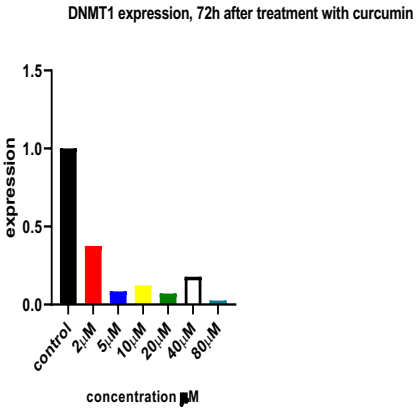

(A)



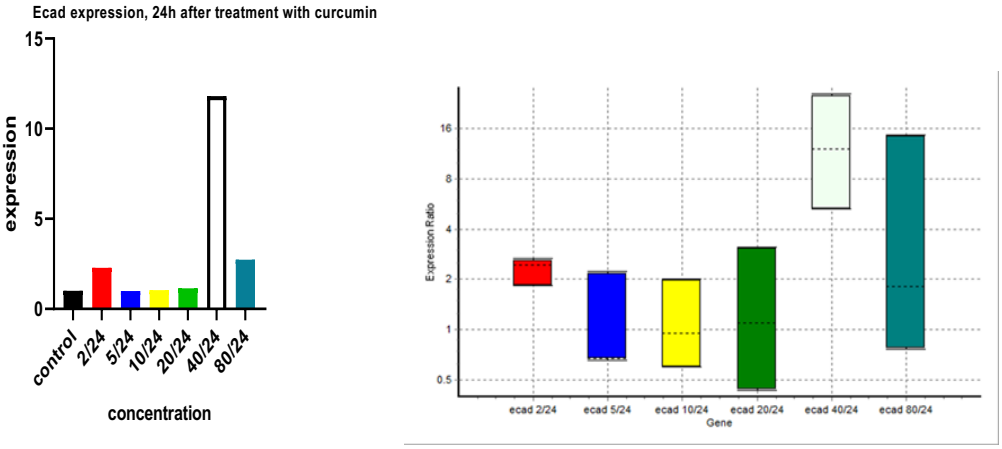

(B)



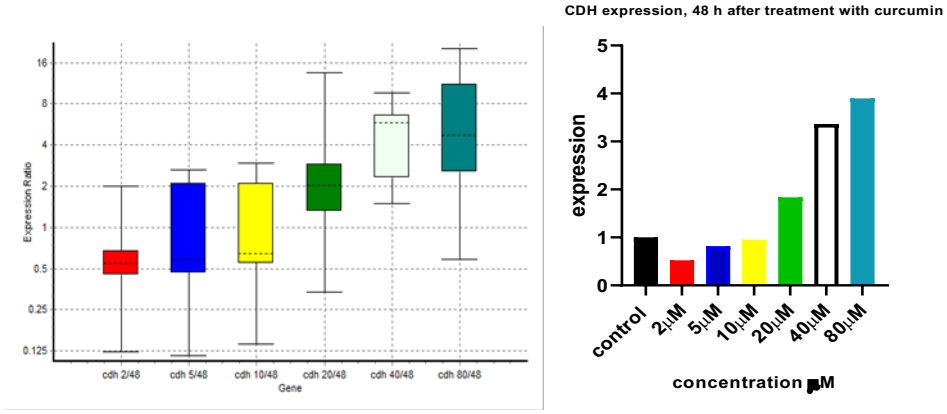




(C)

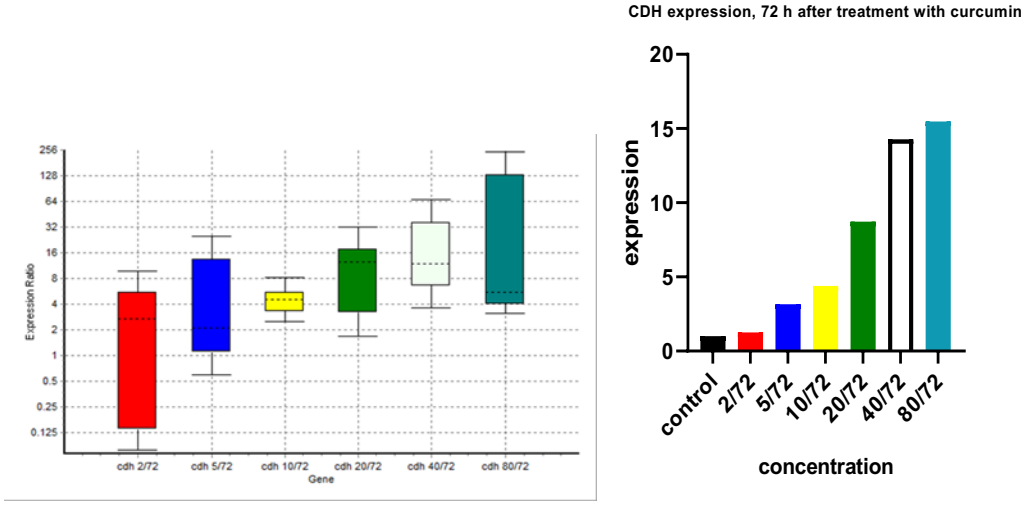
**Figure 2.** Detecting the transcription of DNMT1 mRNA using real-time RT-PCR. MiaPaca-2 cells were treated with 2,5, 10, 20, 40, and 80 μM curcumin for 24, 48, 72 hours.(A) DNMT1 mRNA expression after 24 hrs treated with curcumin (B) DNMT1 mRNA expression after 48 hrs treated with curcumin. (C) DNMT1 mRNA expression after 72 hrs treated with curcumin. The levels of DNMT1 mRNA were analyzed by REST 2009 software ($P< 0.05$)




(A)




(B)




(C)

**Figure 3.** (A) CDH mRNA expression after 24 hrs treated with curcumin (B) CDH mRNA expression after 48 hrs treated with curcumin. (C) CDH mRNA expression after 72 hrs treated with curcumin. The levels of CDH mRNA were analyzed by REST 2009 software ($P< 0.05$)

### 3.4.Discussions

Pancreatic cancer is an aggressive malignancy with an extremely low estimated 5-year survival rate of <2% (Sultana, Smith et al. 2007). In the metastatic stages, pancreatic cancer is never controlled by therapies and chemotherapy drugs, Effectiveness of gemcitabine as a systemic agent in the treatment of advanced stage pancreatic cancer results in a median survival of less than 6 months (Villarroel, Rajeshkumar et al. 2011). This study demonstrated changes in gene expression profiles DNMT1و CDH1 genes in pancreatic cancer cell line in response to exposure to curcumin, that have been reported in previous studies (Shishodia 2013), (Dhillon, Aggarwal et al. 2008).

Antiproliferative effect of curcumin at 20 to 80 μM after 24,48 and 72 hours treatment concentrations of curcumin were seen in pancreatic cancer cell line (MiaPaca-2, respectively ($P<0.05$).

In previous study, has been illustrated inhibitory effects of curcumin observed in G2/M phase cell-cycle arrest and apoptotic cell death in a variety of cancer cells is related to inhibiting NF-κB activation, curcumin has been displayed to suppress the expression of diverse cell survival and proliferative genes, including Bcl-2, Bcl-xL, cyclin-D1and interleukin-6, and accordingly arrest cell cycle, inhibit proliferation, and induce apoptosis (Dhillon, Aggarwal et al. 2008),(Csaki, Mobasheri et al. 2009).

Curcumin substantialy inhibited the proliferation and survival of pancreatic adenocarcinoma, which was related with inhibition of phosphorylation of extracellular receptor kinase (ERK) 1/2, and reduction of protein expression of COX-2 and the EGFR (Zhou, S Beevers et al. 2011).

This study confirms the results of previous research, Our data demonstrated that DNMT1 gene expression is high and CDH1 gene expression is low in cell line MiaPaCa2 that was concordant with prior research. Treatment of Miapaca2 cells with curcumin, leading to downregulation of DNMT1 gene and upregulation of CDH1 gene in this cell line.

According to previous research, Improper CpG island hyper methylation, having been investigated in the early stages of pancreatic cancer and DNMTs protein expression, correlated with advanced stages of the tumor. In addition, high DNMTs protein expression level of patients have been seen to have rather poor survival (Huang, Chou et al. 2019), In most pancreatic cancers, DNMT1 gene expression is increased. DNMT1 gene expression contributes to cell viability, whereas, DNA hyper methylation and over expression DNMT1often inactivates tumor suppressor genes, DNMT1 gene inhibitors have been tested as a treatment for this cancer (Li, Omura et al. 2010).

CDH1 gene expression contributes to maintain an epithelial phenotype, E-cadherin was illustrated to act as an inhibitor of invasion in tumor cell lines and in in vivo tumor models. In humans, There is a strong correlation between CDH1 gene expression and survival of patients (Schmalhofer, Brabletz et al. 2009).

It has been seen in previous studies, CDH1 Gene expression has been decreased in patients with pancreatic cancer by methylation promotor , Silencing or mutation (Ottenhof, De Wilde et al. 2012). Upregulation of DNMT1gene and downregulation CDH1 genes provides conditions for inactivation tumor suppressor genes by methylation and metastasis cancer cells respectively. Based on our in vitro work displayed the activity of curcumin in cell lines of pancreatic cancer,The therapeutic agent of curcumin probably is mediated in part through the antioxidant and anti-inflammatory function of curcumin, Most studies indicate its role in inhibiting cancer tumor growth and metastasis, Curcumin is a safe and non-toxic compound even at a dose of 500-12000 mg and can be used with other anticancer compounds. (von Burstin, Eser et al. 2009, Hamzehzadeh, Atkin et al. 2018). In the future targeting specific gene for identifying people susceptible to cancer and treatment of patient with certain biomarkers, curcumin with its effect on methylation and metastasis can be used as an impressive compound in the prevention and treatment of all types of cancer.

## 4.Conclusions

This study showed that curcumin by reducing the expression of DNMT1 can reduce methylation and also curcumin by increasing the expression of CDH1 can be used as a preventive agent and in the treatment of many cancers.

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