Cyclopamine Decreased the Expression of Sonic Hedgehog and its Downstream Genes in Colon Cancer Stem Cells

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Abstract. *Backround: Most solid cancers including colon cancer are believed to be initiated from and maintained by cancer stem cells (CSCs), that are responsible for treatment resistance, resulting in tumor relapse. The aim of this study was to clarify the possible role of the Sonic Hedgehog (Shh) signaling pathway in the regulation of cancer stem cells. Materials and Methods: The HCT-116 cell line was cultured with fetal bovine serum in RPMI-1640 medium and its sphere was grown in serum-free non-adherent culture. Gene expressions were analyzed by quantitative real-time polymerase chain reaction (qRT-PCR) from cells treated with and without cyclopamine. Results: HCT-116 sphere-derived cells grown in serum-free, non-adherent culture, showed significantly increased expression of stem cell markers, Shh downstream genes and epithelial-mesenchymal transition (EMT) markers compared to parental cells grown in conventional culture. The expression of stemness markers, Shh downstream genes and EMT markers were higher in cancer spheres than the parental cell line and down-regulated by cyclopamine treatment in a dose-dependent manner. Conclusion: Overall, these findings show that cyclopamine treatment could down-regulate the expression of stemness markers, shh downstream genes and EMT markers on HCT-116 spheres.*

Cancer stem cells (CSCs) play an important role in cancer development, because of their main characteristics, which are their self-renewing capacity, chemoresistance, and their tumorigenic capacity (1). They may also play a crucial role in the initiation, progression and recurrence of cancer. Several therapeutic strategies have been suggested to target CSCs. Inhibiting the key signaling pathways that are active in CSCs is one of the most promising strategies for treatment of cancer. Hedgehog signaling (Shh) pathways are essential to regulate the self-renewal of CSCs and are aberrantly activated in a variety of cancers.

The classic inhibitor is cyclopamine, a naturally-occurring teratogenic alkaloid. It disrupts cholesterol bio-synthesis and specifically antagonizes the Shh signaling pathway through direct interaction with Smoothened (*SMO*), which functions upstream of *GLI1* (2).

Epithelial-mesenchymal transition (EMT) is a process in which cells lose their epithelial character and acquire a migratory mesenchymal phenotype (3). EMT is induced by transforming growth factor-β1 (TGF-β1), that is involved in the promotion of tumor invasion and metastasis. It is also closely-related to drug resistance of tumor cells (4). Loss of E-Cadherin, which is a main determinant of epithelial tissue organization and cell polarity, is considered a hallmark of EMT (5). Recent studies show a repression of E-Cadherin related to enhanced invasiveness and metastasis, while conversely, up-regulation of E-Cadherin decreases in tumor malignancy (6, 7).

To eradicate colon cancer and prevent recurrence, it is imperative that HCT-116 CSCs should be specifically and efficiently inhibited. In addition, CSCs undergoing metastasis often express epithelial-to-mesenchymal transition and the high EMT markers have been observed in dissemination of carcinoma cells from primary epithelial tumors. The interaction between stemness characteristics and EMT expression has advanced results in recent studies. Researchers have shown that EMT acquires CSC properties, increases cancer cell tumorigenicity and shows a crucial link to metastasis.

The cancer-preventive effects of cyclopamine are widely supported by results from epidemiological, cell culture, animal and clinical studies. This property has been mainly attributed to the capacity in inhibiting cancer growth in

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Key Words: Cancer stem-like cells, epithelial-mesenchymal transition, cancer cells, sonic hedgehog.

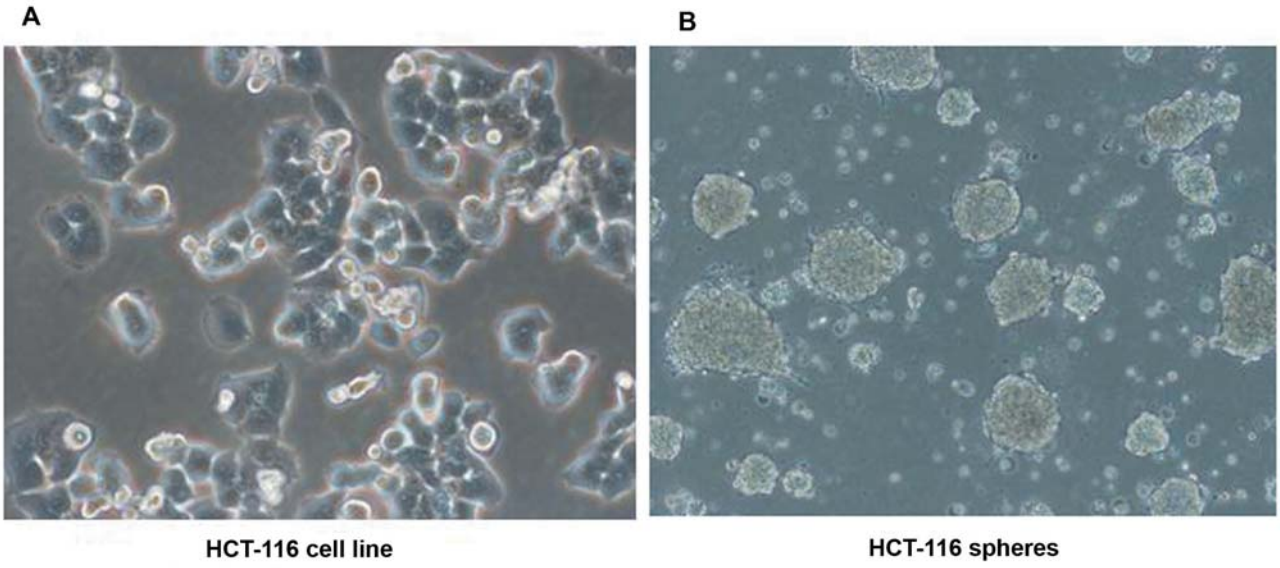

Figure 1. *Representative images of HCT-116 colon cancer cell line. A) HCT-116 colon cancer cell line B) HCT-116 spheres.*

various cancers by several mechanisms. CSCs are believed to be more drug-resistant compared to parental cells and so can be ultimately inducing tumour recurrence after the completion of treatment. Thus, removal of CSCs becomes more and more crucial to chemotherapy and drugs that selectively target CSCs offer a greater promise for cancer treatment. The aim of this study was to determine the mRNA expression levels of stemness markers, Shh downstream genes and EMT markers on HCT-116 cancer sphere and to explore the possible role of cyclopamine on the expression of these genes.

The goal of this study was to clarify the possible role of Shh signaling pathway's genes in the regulation of CSCs-, and effect of cyclopamine to EMT, tight junction proteins, and CSCs markers.

Materials and Methods

Cells and culture conditions. The human colon cancer (HCT-116) cell line was bought from the American Type Culture Collection (ATCC, 10801 University Boulevard Manassas, VA, USA). The cell line was maintained as a monolayer in Roswell Park Memorial Institute medium 1640 (RPMI 1640) (Invitrogen, Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen/Gibco-), 100 IU/ml penicillin G and 100 μg/ml streptomycin at 37˚C in a humidified 5% CO_2 incubator. The cells were collected and washed to remove serum, then suspended in serum-free Dulbecco modified Eagle's minimal essential medium (DMEM/F12) (Invitrogen/Gibco-,) supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 20 ng/ml human recombinant epithelial growth factor (hrEGF) (BD Biosciences, cat.n: 354052, San Diego, CA, USA), 10 ng/ml human recombinant basic fibroblast growth factor (hrbFGF) (BD Biosciences, cat.n: 354060-), 2% B27 supplement without vitamin A, 1% N2 supplement (Invitrogen,).

Isolation of RNA. Total RNA was extracted from HCT-116 parental cells and its sphere-, using TRIzol reagent (Invitrogen). cDNA was prepared using the high capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster, CA, USA), following the manufacturer's instructions. The RNA pellets were stored at –80˚C until use.

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). QRT-PCR was performed with SYBR Green master mix real-time core reagents on an ABI 7500 (Applied Biosystems-,) according to the manufacturer's instructions. Primers for qRT-PCR were as follows: Taqman® stemness genes: Oct-4 (*POU5F1*) Hs03005111_g1; *NANOG* Hs02387400_g1; CD-44 Hs01081473_m1; *EpCAM* Hs00901885_m1. Shh pathway downstream genes: *PTCH1* Hs00181117_m1; *SMO* Hs01090242_m1; and Shh downstream gene Gli1 Hs01110766_m1. Tight junctions are Claudin-4 (*CLDN4*) (Hs00533616_s1) and Occludin (OCLN) Hs00170162_m1.

Cyclopamine treatment. The HCT-116 cell line was plated in 10 cm dishes and incubated for 48 at 37˚C, in 5% CO_2 and 95% humidity. Then the cells were transferred into sphere forming medium and incubated for 12 days at 37˚C, in 5% CO_2 and 95% air. The sphere cells were exposed to cyclopamine at 2 μM and 5 μM or no cyclopamine as a control group for 48 h. Lastly, the cells were harvested and subjected to RNA extraction.

Statistical analysis. Data were obtained from experiments performed at least 3 times with a minimum of triplicates. All values in the figures and text are shown as means±standard deviation (SD). Statistical analyses were performed using the Stat.View (SAS

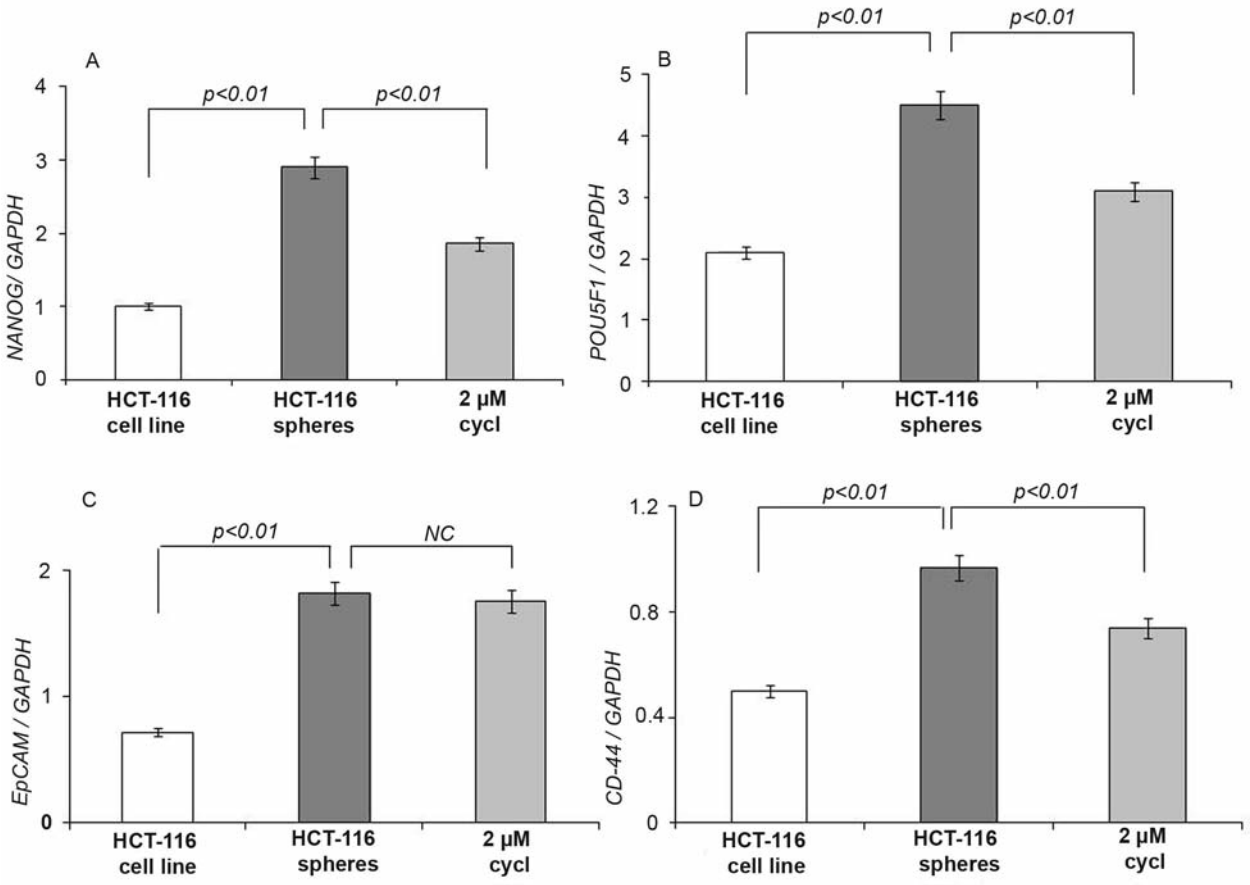

Figure 2. *mRNA expression level stemness markers before and after cyclopamine treatment in HCT-116 spheres. A) The mRNA level of NANOG was significantly expressed in HCT-116 spheres compared to HCT-116 cell line. After cyclopamine treatment the mRNA was down-regulated in HCT-116 spheres. B) The mRNA level of POU5F1 was significantly expressed in HCT-116 spheres compared to HCT-116 cell line. After cyclopamine treatment the mRNA level was down-regulated in HCT-116 spheres. C) The mRNA level of EpCAM was significantly expressed in HCT-116 spheres compared to HCT-116 cell line. After cyclopamine treatment the mRNA level presented no difference in HCT-116 spheres. D) The mRNA level of CD-44 was significantly more expressed in HCT-116 spheres compared to HCT-116 cell line. After cyclopamine treatment the mRNA was down-regulated in HCT-116 spheres.*

Campus Drive, Cary, NC, USA). Any significant differences among mean values were evaluated by the student's *t*-test. A *p*-value of less than 0.05 was accepted as statistically significant.

Results

mRNA level of surface marker and stemness gene before and after administration of cyclopamine in HCT-116 sphere. HCT-116 sphere succesfully formed from HCT-116 parental cells (Figure 1A and B). The stemness genes (*NANOG, POU5F1*) and surface markers (*CD-44, EpCAM*) were significantly expressed in HCT-116 sphere compared to HCT-116 colon cancer cell line ($p<0.01$). After administration of cyclopamine the mRNA level of *NANOG, POU5F1* and CD-44 was reduced in HCT-116 sphere ($p<0.01$) (Figure 2 A, B, C). However, the mRNA level of EpCAM was not changed after administration of cyclopamine (Figure 2C).

Regulation of mRNA level of Shh downstream genes by cyclopamine in HCT-116 sphere. As indicated on Figure 3 (A-C), a significant mRNA expression level of *PTCH1, SMO* and *GLI1* was observed between HCT-116 sphere and its cell line ($p<0.01$). After treatment of cyclopamine the mRNA level of these genes was significantly reduced.

mRNA expression level of the epithelial marker E-Cadherin and mesenchymal marker Vimentin, before and after cyclopamine treatment. As shown in Figure 4 A and B, the mRNA level of *CDH1* (E-Cadherin) was down-regulated in HCT-116 sphere compared to HCT-116 cell line, while the mRNA level of *VIM* (Vimentin) was overexpressed in HCT-116 sphere than in the HCT-116 cell line. After cyclopamine treatment the mRNA level of CDH1 (E-Cadherin) was up-regulated in HCT-116 sphere, whereas

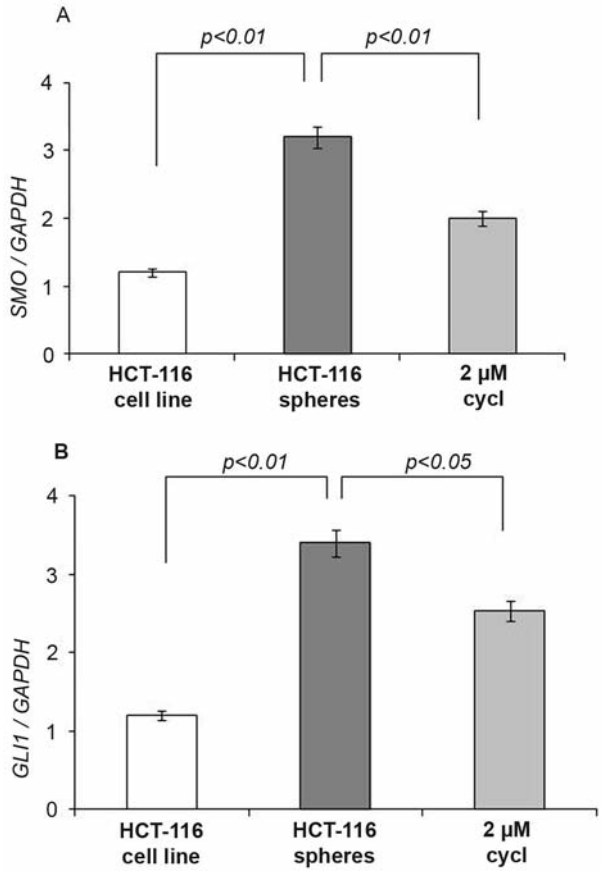

Figure 3. *Shh inhibitor suppresses mRNA levels of SMO and GLI1 in HCT-116 spheres. A) SMO expression before and after administration on 2 μM dose in HCT-116 spheres. B) GLI1 expression before and after administration on 2 μM dose in HCT-116 spheres.*

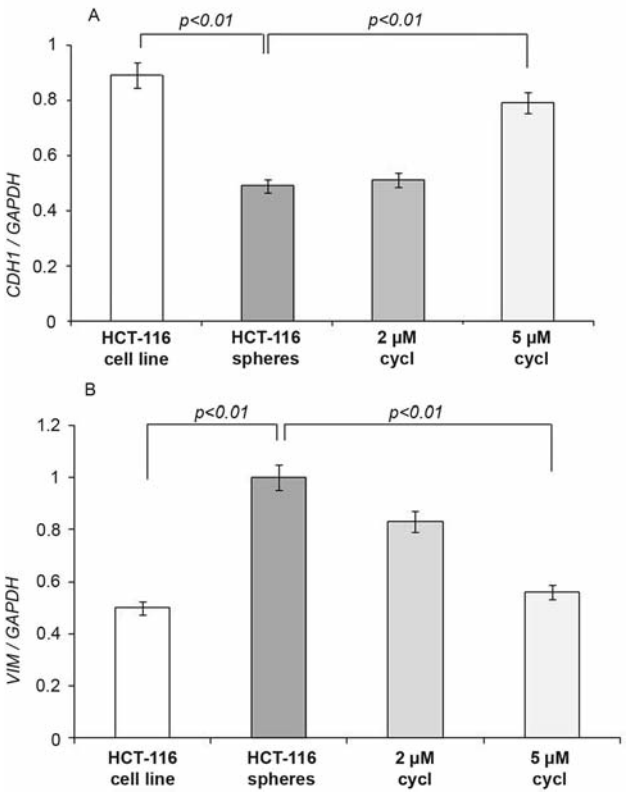

Figure 4. *Shh inhibitor (cyclopamine) suppresses expression of EMT markers in HCT-116 spheres. A) mRNA level of CDH1 (E-Cadherin) significantly expressed in HCT-116 cell line than HCT-116 spheres. After administration of cyclopamine on 2 μM and 5μM dose the mRNA level was up-regulated in HCT-116 spheres. B) mRNA level of VIM (Vimentin) significantly expressed in HCT-116 spheres than HCT-116 cell line. After administration of cyclopamine on 2 μM and 5 μM dose the mRNA level was down-regulated in HCT-116 spheres.*

the mRNA level of *VIM* was down-regulated in HCT-116 sphere ($p<0.01$).

mRNA expression level of tight junction proteins in HCT-116 sphere. The mRNA expression level of CLDN4 (claudin-4) and OCLN (occludin) were significantly expressed in HCT-116 sphere than the HCT-116 cell line. After administration of cyclopamine the mRNA level was down-regulated in HCT-116 sphere ($p<0.01$).

Discussion

Colon cancer sphere of HCT-116, grown in serum-free non-adherent culture, showed increased expression of stem cell markers such as *NANOG, POU5F1, CD-44* and *EpCAM*, compared to parental cells grown in conventional cultures. Previous studies identified the expression of cell surface markers *CD-44, CD24, ESA, CD-133* (8)- and *EpCAM* (9) to be highly expressed in cancer sphere. *POU5F1* and *NANOG* are essential regulators of stemness by promoting proliferative potential and self-renewal in embryonic stem cells during early embryonal development. These are specifically expressed in the human embryonic pluripotent stem cells (10). Down-regulation of stem cell markers may be a novel approach for colon cancer therapy, especially for HCT-116 CSCs. In the present study, we have convincingly demonstrated that cyclopamine treatment down-regulated the stemness markers, *NANOG, POU5F1* and *CD-44*, which indicates that cyclopamine could be an effective anticancer agent for HCT-116 spheres.

Several therapeutic strategies have been suggested to target CSCs. Inhibiting the key signaling pathways that are active in CSCs is one of the most promising strategies for treatment of cancer. Shh pathways are essential to regulate

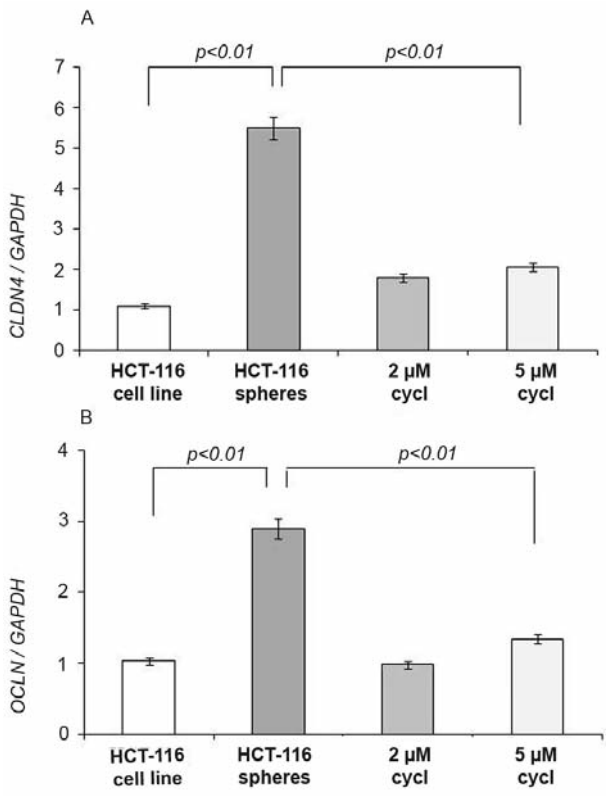

Figure 5. *Cyclopamine influences in tight junction markers in HCT-116 spheres. A) mRNA level of CLDN4 (Claudin-4) decreased after administration of cyclopmine in HCT-116 spheres. B) mRNA level of OCLN (Occludin) decreased after administration of cyclopmine in HCT-116 spheres.*

the self-renewal of CSCs and play important role in maintaining stemness by regulating the expression of stemness genes (11). Mueller *et al*. reported a high expression of CSC markers correlated with Shh up-regulation in gemcitabine-resistant pancreatic cancer cells (12) explaining that the Shh pathway target genes could induce self-renewal, survival, and migration of cancer progenitor cells (13, 14).

Zhang *et al*. reported that the *Shh, PTCH1* and *GLI3* were more highly expressed in $CD44^{+}CD24^{+}$ gastric cancer stem cells, than cancer cells (15), while cyclopamine treatment was shown to inhibit up-regulation of these genes by interfering with activation of *SMO* (16, 17). In line with this, in the present study, the mRNA level of Shh downstream genes, *PTCH1* and *SMO* were significantly overexpressed in HCT-116 sphere, than the HCT-116 cell line, with cyclopamine treatment inhibiting up-regulation of these genes in HCT-116 sphere.

The Shh signaling pathway activation includes epithelial-mesenchymal transition (EMT), which is required for migration of Shh-activated cells during tissue morphogenesis. To eradicate colon cancer and prevent recurrence, it is imperative that colon CSCs should be specifically and efficiently inhibited. In addition, CSCs undergoing metastasis often express epithelial-to-mesenchymal transition and the high EMT markers have been observed in dissemination of carcinoma cells from primary epithelial tumors. The interaction between stemness characteristics and EMT expression has advanced results in recent studies. Researchers have shown that EMT acquires CSC properties, increases cancer cell tumorigenicity and shows a crucial link to metastasis.

In the present study, the mesenchymal marker Vimentin was up-regulated in HCT-116 sphere, while epithelial marker E-Cadherin was down-regulated in HCT-116 sphere. After using cyclopamine the Vimentin was down-regulated and E-Cadherin was up-regulated in HCT-116 spheres. E-Cadherin has a down-regulated expression upon an increased expression of Shh/Gli1 (18). E-Cadherin plays a main role in the development of epithelial tissue and cell polarity (19) and its loss is a hallmark of an EMT. A previous study mentioned down-regulation of *GLI1* and showed up-regulation of E-Cadherin in gastrointestinal neuroendocrine carcinomas occurring after the use of Shh inhibitor cyclopamine (20). The results of the present study reveal that cyclopamine could influence the production of EMT by *GLI1* down-regulation, which could activate or increase mRNA level of *CHD1* (E-Cadherin) in a colon cancer sphere of the HCT-116 cell line.

In addition, tight junction proteins showed high expression of EMT genes in human breast cancer (21). Taube and Turksen reported the low or nil expression of *CLDN4* (claudin-4) that has been associated with breast cancer-derived CSCs (22, 23). However, there is a high expression of claudin-4 in ovarian CSCs (24). In our study high expression of claudin-4 and occluding in HCT-116 sphere was significantly down-regulated by cyclopamine treatment. The expression of tight junction proteins is controversial in CSCs. Future studies need to investigate the role of tight junction proteins in CSCs in depth.

In conclusion, overall, our findings show that HCT-116 cells with sphere formations possess the properties of CSCs. Using this model, we found that cyclopamine treatment down-regulated genes associated with self-renewal and invasive capacities in HCT-116 spheres. This supports the notion that cyclopamine may decrease recurrence and metastasis in colon carcinoma.

Conflicts of Interest

Bat-Erdene Batsaikhan and other co-autors declare no conflict of interest.

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