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Original Article:

5-Azacytidine Enhancing Expression of E-cadherin in Adenocarcinoma Cell Line

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Abstract

Introduction: In this study, we assessed the expression of E-cadherin in HT29 cell line treated with 5-Azacytidine and colorectal cancer patient in an Iranian population. E-cadherin expression promotes metastasis and prognosis of colorectal cancer (CRC). 5-Azacytidine, a DNA methyl transferase inhibitor, is a clinically used epigenetic drug for treatment of cancer including colorectal cancer, leading to genes activation involved in tumor suppression, especially E-cadherin.

Materials and Methods: HT29 cell line treated with 5-Azacitidine and 40 polyps, 20 tumors and 40 adjacent normal tissues samples were enrolled in this study. Using the real-time PCR method, the expression levels of E-cadherin were examined in treated cell line and colorectal cancer tissue.

Results: This study proves that 5-Azacytidine induces over expression of E-cadherin in adenocarcinoma cell line, while the expression levels of E-cadherin were not different in tumor and polyp than adjacent normal tissue.

Conclusion: To conclude, 5-Azacytidine induces re-expression of E-cadherin in adenocarcinoma cell line. Thus, 5-Azacytidine as demethylation drug activated tumor suppressor gene as E-cadherin.

Keywords: 5-Azacytidine, Colorectal cancer, E-cadherin, Polyps

1. Introduction

Colorectal cancer (CRC) is a common and heterogeneous disease, which arises from the accumulation of genetic alternation. The etiology of CRC is very complicated and associated with different genetic and epigenetics features [1-3].

Since the clinical course of primary CRC in each patient is different from other patients, determining the outcome of each patient is not known; thus, identifying the factors contributing to prediction of the CRC, is useful in prognosis and treatment [4].

Several studies have aimed at the identification of expression levels of gene to discriminate malignant colorectal lesions from benign ones [5-7]. Most of such studies have focused on the expression analysis of protein coding genes with putative roles in the tumorigenesis and metastasis processes [8, 9].

Different gene patterns in determining the prognosis and predicting patients' response to treatment regimens in various cancers are used, including E-cadherin [10, 11]. Some studies have reported an inverse relationship between E-cadherin expression and tumor progression; the loss of E-cadherin expression promotes metastasis [12].

As tumor progresses to metastatic malignancy, the cancer cells gain the ability to invade distant tissues. Metastasis occurs when it migrates from an initial site to a secondary one. Hence, epithelial tumor cells undergo phenotypic changes and metastasize. The epithelial to mesenchymal process (EMT) leads to metastasis. Epithelial cells are closely related to surrounding cells, and the cells maintain polarity and play an important role as a barrier [13, 14].

Reduced levels of the cell-cell adhesion molecule E-cadherin are related with loss of differentiation in a number of human cancer including CRC [15-17].

The E-cadherin gene (CDH1), which is almost 100 kb long, is housed on chromosome 16q22.1. The gene region includes 16 exons, sized between 115 and 2245 bp and interfered by 15 introns in total [18].

CDH1 gene transcripts E-cadherin protein into a precursor polypeptide of 135 kDa.

The matured E-cadherin protein is a 120 kDa, Ca2+-dependent transmembrane glycoprotein. The amino terminal of E-cadherin part five extracellular cadherin domains between which Ca2+ ions bind and its adhesive activity lies [18].

E-cadherin plays a main role in epithelial cell adhesion and the loss of its function is a key contributor to cancer progression because the majority of solid tumors are carcinomas originating from epithelial tissue [5, 6]. 5-Azacytidine, a DNA methyl transferase inhibitor, is a clinically used epigenetic drug for cancer therapy leading to re-expression of silenced genes and changing expression of genes involved in tumor suppression especially E-cadherin [19, 20].

In this study, we evaluated the expression of E-cadherin in HT29 cell line treated with 5-Azacytidine and colorectal tissue patient in an Iranian population for the first time.

2. Materials and Methods

HT29 cell line culture

For examining the effect of 5-azacytidine epigenetic drug on HT29 cell line and the changes in E-cadherin expression cell culture were performed. HT29 cell line deriving from adenocarcinoma cells was used. This cell line was purchased from the Pasteur Institute of Iran. The culture medium used RPMI containing 5% FBS, 1% essential amino acid (NEAA, Gibco) and 1% antibiotic. Cells were transplanted for several generations in special flasks using trypsin (0.25%) EDTA. To find the right concentration of drug treatment, the cells were placed in the cavity at three times 24h, 48h and 72h 96 houses. In order to confirm the result, this test was performed in three replications for each hole. After placing the appropriate number of cells (10,000 cells) in the plate cavities of 96 cells and treatment with different concentrations of 5-azacytidin, from a concentration of 160 µM to a concentration of 1 µM, serial dilution was performed. In the incubator at 37° C and 5% Co2 were incubated and after incubation time (24h, 48h and 72h), the plates were removed from the incubator and the lethality of the drug was examined using MTT method by ELISA reader at 570 / 630nm. Cells were grown in culture medium containing different time and concentrations of 5-Azacitidine (Sigma, UK, Cat No: 24890525) to select the appropriate amount of drug. Finally, the weight ratios of 1.25 and 10 µM were selected as the optimal ratio. One of the wells was used as a control. After 48 hours (proper time), the cells were isolated and mixed with 200 µl of PBS, and then RNAs were extracted to calculate the expression of genes before and after treatment.

MTT assay

To determine the viability of cells, MTT method (Sigma, UK, Cat No: 298-93-1) was used. MTT powder with a concentration of 5 mg / ml was dissolved in physiological serum. First, 1.5. 10^5 cells per milliliter per house were poured from 96 house culture plate and the concentration of the desired substance was adjusted. At the end of the time intervals (24, 48 and 72 hours) of cell treatment with 5-azacytidine, 100 µl of MTT solution was added to each house and incubation was performed for several hours at 37 ° C away from light. After incubation, the culture medium was drained, then, 1000 µl (Merck, Germany, Cat No: 1096780100) of DMSO was added to each well to completely dissolve the Formazan crystals. The insoluble crystals of formazan were dissolved and the maximum optical absorption (OD) was measured at 570 nm and the percentage of living cells was calculated.

Study population

The study population consisted of 20 tumor- and 40 polyp-samples in the intestine with 40 adjacent normal tissues. These people are selected from those who were referred to the Research institute for Gastroenterology and Liver Diseases (RIGLD) of Shahid Beheshti University of Medical Sciences Tehran, Iran between December 2018 and June 2019. All the participants were well-informed about the tests progress and their clinical data were obtained by reviewing and completing a standard questionnaire application. Written consent was obtained from the

participants in the study. Tissue specimens were sampled from all patients on whom colonoscopy was performed. Expert pathologist confirmed the tumor or polyp. The demographic data of the patients, such as age, sex, weight, body mass index (BMI), and familial history of CRC, were taken from the clinic records. The methods were approved by the RIGLD's ethics committee. (Study Ethics Code 1392/704)

Samples included fresh tissue from intestinal appendages including primary polyps as well as fresh tissue from intestinal cancerous tumors. Normal tissue was also removed from the intestine at a distance of 1 to 10 cm.

In this study, the inclusion criteria were: (1) patients being originally from Iran, and (2) cases with positive pathology results; the exclusion criteria were: (1) individuals that underwent colonoscopy, but the biopsy was not performed completely, (2) those who did not have valid clinical and pathological data, and (3) RNA or DNA samples that did not have the good quality and concentration.

RNA extraction and real-time PCR analysis

Total RNA was extracted from the tissue of the patients and cell line using the Total RNA Extraction Kit (Qiagene, Germany, Cat No./ID: 74104), as per the manufacturer's instructions. The RNA concentration was measured by a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies) and its quality was assayed by measuring the A260/A280 ratio, in the range of 1.8-2.0.

One µg of the total RNA was reverse-transcribed with the help of the RevertAid RT Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

The expression level of genes was evaluated by quantitative Real-time RT–PCR with SYBR Premix Ex Taq (TaKaRa Bio, Kyoto, Japan) on Rotor-Gene system (Qiagen, Hilden, Germany). Primers were designed using Primer 3 software (Forward: 5'_ CAGAATGACAACAAGCCCGA _3', Reverse: 5'_ ATGGCGGCATTGTAGGTGTT _3'). The procedure for qRT-PCR was as what follows: 30 s of pre-denaturation at 95°C, followed by 40 cycles of two-step PCR denaturation at 95°C for 5 s and annealing extension at 60°C for 34 s. Human GAPDH and β-globin genes was served as the endogenous control. The relative expression was normalized to the reference gene, using the Pfaffl method. The Real-time PCR was repeated three times for each sample.

Statistical analysis

In order to evaluate the expression levels of E-cadherin among the target subjects, the Relative Quantitative (RQ) qPCR technic was used. In this method, the expression value of a gene is shown as the fold change. The values that are less than 0.5 mean decrement depicts no change. The values which are higher than 2 illustrate the increment in the expression of the target genes as compared to the control. All the data are represented as the mean ± S.D (standard deviation) and executed using the GraphPad Prism software (version 6, USA). Statistical significance was achieved when $P < 0.05$.

3. Results

E-cadherin expression levels before and after treatment 5-Azacytidine

The expression of the E-cadherin in the samples was examined before and after the treatment. The expression of E-cadherin gene in the samples treated with 5-Azacytidine increased compared to the untreated state, which indicates the effect of this drug on the expression of this gene. (p<0.00) Interestingly, the rate of expression in drug-treated samples at a concentration of 1.25 µl showed a more increase in expression than samples treated at a concentration of 10 µl.

E-cadherin expression levels in Study population

We evaluated the expression levels of the E-cadherin in the samples by qRT-PCR. Our results demonstrated no significant changes in both colorectal cancerous and polyp tissues in comparison with their corresponding normal tissues (P> 0.05). (Table 1).

Table 1. Expression levels of E-cadherin in tissue samples

Refrence gene	Polyp/normal		Tumor/Normal		Tumor/polyp	
B-glubin/ E_cadherine	Expression 0.986	P value 0.982	Expression 0.948	P value 0.966	Expression 0.482	P value 0.568
GAPDH/E_cadherine	Expression 1.874	P value 0.535	Expression 0.803	P value 0.893	Expression 2.755	P value 0.550

4. Discussion

Several transcription factors are involved in EMT, one of the most important of which is ZEB1 / ZEB2 that can decrease expression of E-cadherin. E-cadherin causes epithelial cell adhesions and E-cadherin

aberrant expression promotes tumor progression[14]. Loss of *E-cadherin* is used for the diagnosis and prognosis of epithelial cancers including colorectal cancer. Lu and colleagues showed that suppressors of E-cadherin promoter increase EMT and promote cancer cell growth, migration, and progression of colon cancer [21]. 5-azacitidine epigenetic drug increase the expression of E-cadherin in cell line [22]. Also, several reports have shown that 5-azacitidine induces cell death and inhibits metaststsis in tumor cells [23, 24] by altering the epigenetic state, but the details of 5-azacitidine-induced cell cycle arrest and inhibition of metastasis are not completely understood. In the present study, E-cadherin expression increased in HT-29 adenocarcinoma cells after treatment with 5-azacitidine demethylation drug. In agreement with the present study, Wang et al. reported E-cadherin gene mRNA expression increased after treatment with 5-azacitidine in rat epithelial cell line [22]. Also, Yamanaka et al. reported that methylation in the promoter of the E-cadherin gene reduces its expression and increases EMT and invasion where metastasis of tumor cells occur [25]. Therefore, demethylation by the 5-azacitidine epigenetic drug can increase the expression of E- cadherin [22]. In most cancers, decreased expression of miR-200 is associated with methylation of CpG island promoter increasing E-cadherin expression. Thus, 5-azacitidine demethylated miR-200 and E-cadherin promoter and suppressed EMT and metastasis [26].

Our results have shown that there were no significant associations between the expression of E-cadherin gene in tumor and polyp tissue compared to adjacent normal tissue. Also, Ghadad et al. reported that E-cadherin expression was normal in 47% of patients with lung cancer [27]. On the other hand, a decrease in the expression of E-cadherin in patients with colorectal cancer has been reported [16]. Down-regulation of E-cadherin gene expression was reported in pancreatic cancer cells [28]. E-cadherin expression regulation , suppressing EMT in colon cancer, can prevent the progression and proliferation of this cancer [21]. In colorectal cancer suppression of E-cadherin expression has been reported to increase the expression of mesenchymal markers as well as cell migration. Abnormal hypermethylation in the promoter plays a key role in cancer by silencing tumor-suppressor genes. Especially, downregulation of tumor-suppressor genes, such as E-cadherin associated with aberrant methylation in cancer cell lines and primary tumors. Increased expression of E-cadherin significantly reduces EMT and sensitivity to treatment, especially in primary lymph node-derived cancer cells [29].

E-cadherin involved in cell adhesion and migration can be biomarker in metastasis of cancer. There are a number of studies which correlate the expression of E-cadherin and cancers. However, no recent study has reported a significant difference between E-cadherin gene expression level in tumor/ polyp tissue and adjacent normal tissue. This can be at least partly due to the small sample size. However, in order to be able to talk about this molecule and its association with colorectal cancer, further research is needed. Of course, genes in different populations and cancer show diverse behaviors, which warrants further research. This study proves that the expression levels of E-cadherin are not different in tumor and polyp than adjacent normal tissue. Yet, treatment with 5-azacitidine increase the expression of E-cadherin in adenocarcinoma cancer cell line.

5. Conclusion

In conclusion, our study showed E-cadherin expression increased while treated with 5-azacitidine demethylation drug and was re-activated by methylation inhibitor 5-azacitidine; this provides a new insight into the therapeutic strategy of colorectal cancer.

Ethical Considerations

Compliance with ethical guidelines

All procedures performed in studies involving human participants were in accordance with the ethical standards of the Gastroenterology and Liver Disease Research Centre at Shahid Beheshti University of Medical Science, Tehran, Iran.

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Author's contributions

Conceptualization, S.S., Sh.I and H.A.A.; methodology, S.S., R.M., M.A., and M.A.B.; software, S.S. validation, S.S., H.A.A., M.A. and R.M.; formal analysis, S.S; investigation, S.S, R.M., M.A. and H.A.A.; resources, H.A.A.; data curation, S.S., and H.A.A.; writing—original draft preparation, S.S. and Sh.I; writing—review and editing, S.S. and M.A

Conflict of interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the

collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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