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Research Article

CDH1 Gene Expression Alterations in Prostate Cancer Cell Lines After DAC And TSA Applications

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Abstract

Cancer is characterized by genetic and epigenetic disorder by uncontrolled growth and deterioration of the behavior of normal cell division (proliferation) and spread to other tissues (metastasis). Genes that control the division patterns in cancer cells has been mutated or show anormal expression levels or profiles. Prostate cancer is formed by uncontrolled proliferation of cells in the prostate gland. The expression of one of the major intercellular epithelial cell adhesion protein, e-cadherin, mediates the epithelial cell-cell interactions through calcium-dependent homophilic interaction of its extracellular domain. However, a reduction or loss of membranous expression of e-cadherin protein has been reported in invasive cancer cells at their primary sites. In this study, DNA methyl transferase inhibitor [5-aza.2.deoxycytidine (DAC), an epigenetic modulating drug that can reverse DNA methylation] and histone acetyltransferase enzyme inhibitor (TSA: Trichostatin A) applied to prostate cell lines (LNCaP, PC3 ve DU145) to determine gene expression levels of *CDH1*. When the changes in *CDH1* mRNA level were examined after incubation of DU145 cells with 48 h 2 µM DAC, 300 nm TSA and 42 h DAC + 6 h 300 nm TSA, significant upregulation was detected in gene expression compared to control in all three applications ($P < 0,001$). When changes in *CDH1* mRNA level in PC3 cells were examined; significant upregulation was determined in 2 µM DAC application, while significant downregulation was determined in 500 nm TSA and 42sa DAC + 6 h 500 nm TSA applications ($P < 0,001$). When the changes in *CDH1* mRNA level of LNCaP cells were examined, significant upregulation was determined in 1.8 µM DAC application, while significant downregulation was determined in 200 nm TSA and 42sa DAC + 6 h 200 nm TSA applications ($P < 0,001$).

As a result, prostate cancer cell lines may be an ideal medium for testing demethylation drugs, but it is uncertain whether *CDH1* promoter methylation is a specific mechanism for e-cadherin suppression. Therefore, in some cases, failure of the clinical activity of demethylation agents can be observed in the presence of methylated genes.

Keywords: e-cadherin; Prostate Cancer; DU145; LNCaP; PC3; Cell Line

Abbreviations

CDH1: Cadherin 1 Gene; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase; DAC: 5-aza.2.deoxycytidine; TSA: Trichostatin A; HDAC: Histone Deacetylase; HDACi: HDAC Inhibitors; HAT: Histone Acetylases; DNMT: DNA Methyl Transferase; CT: Treshold Cycle; IC50: Half-maximal Inhibitory Concentration

Introduction

Cancer is a genetic and epigenetic disease characterized by the disruption of the division pattern behavior of normal cells, their uncontrolled proliferation (proliferation) and spread to other tissues (metastasis). The genes controlling the division pattern in cancer cells are either mutated or show an abnormal expression profile. Ultimately, the combination of loss of control of cell cycle checkpoints and metastatic spread creates cancer cells. As is known, a single mutation in normal cells is not sufficient to carry out cancerous malignant cell formation and tumorigenesis [1].

It is known that epigenetic mechanisms play a role in the development of prostate cancer, which is generally accepted as a genetic disease. Epigenetic modifications are very important in many diseases, including cancer. Various mechanisms are involved in the regulation of gene expression. One of them is the epigenetic control mechanism [2]. Epigenetic changes are regional and transient changes in the expression of a gene of interest without any change in DNA sequence [3] and are responsible for the stability of chromatin structure, genome integrity, regulation of tissue-specific gene expressions, embryonic development, suppression of intragenomic parasites, genomic imprinting, and inactivation of the X chromosome [1,4]. Epigenetic programming is very important for mammalian development, and its stable inheritance is very important to maintain specific functions according to tissue and cell type.

Accelerated studies of epigenetic mechanisms have expanded knowledge of epigenetic regulation, including long-distance chromatin remodeling, DNA methylation, transtranslational histone modifications, and the involvement of small and long non-coding RNAs [5].

DNA methylation and histone modifications are among the most studied epigenetic mechanisms [6]. Methylation of CpG islands located in the control regions of genes completely or partially stops the transcription of these genes. However, a second epigenetic mechanism that causes suppression of gene expression is histone modifications. Among histone modifications, Histone 3 (H3) deacetylation, H3 lysine 4 (H3K4) demethylation, and H3 lysine 9 (H3K9) methylation are markers for heterochromatin regions [2].

The *CDH1* gene expresses the e-cadherin protein. E-cadherin, one of the most important intercellular epithelial cell adhesion proteins, regulates epithelial cell-cell interaction through calcium-dependent homophilic interaction of its extracellular domain. Therefore, reduction or loss of e-cadherin protein has been reported in invasive cancer cells and primary sites of cancerous cells [7-11]. The results of this study show that the expression of e-cadherin is important for the cellular differentiation and polarity of normal epithelial cells in tissue, and the loss or reduction of its expression in invasive cancers results in cell migration and intercellular separation from primary sites, which is the possible initial event in the metastasis process.

Reactivation of the e-cadherin gene in cancer cell lines was carried out with the demethylating agent 5-aza.2.deoxycytidine, indicating the role of hypermethylation in the suppression of e-cadherin expression in these cell lines [8-11].

Histone deacetylases (HDACs) exist as large, repressor multiprotein complexes that mediate acetyl group removal from lysines on histone tails. To date, 18 HDACs have been identified in mammals [12]. HDACs have increased expression in many types of cancer, including ovarian, breast, bladder, and other cancers, and are thought to promote carcinogenesis through key transcriptional interactions and acetylation [13]. Therefore, HDAC enzymes are identified as attractive targets for cancer therapy.

HDAC inhibitors (HDACi) are natural or synthetic chemical compounds with extensive functions in the cell. Various HDACi have been designed to target the catalytic domains of HDACs. HDACi contains many clinical drugs with a broad therapeutic spectrum and is under investigation for use in cancer therapy. Thus, HDACi can alter the balance between histone acetylases (HAT) and HDACs, resulting in the accumulation of acetylated histones/non-histone proteins that cause transcriptional and related molecular effects. Based on their structure and specificity, HDACi's can be divided into several classes, including hydroxamates, cyclic peptides, aliphatic acids, and benzamides. As we know, class I, II and IV HDACs share

zinc-dependent homologies. Therefore, many inhibitors are non-specific and can be used to inhibit multiple isoforms of HDACs. TSA was the first natural hydroxymate discovered with HDAC inhibition function in 1990 [14]. TSA can inhibit both class I and II HDACs. Vorinostat is TSA-like in structure and is the newest HDACi approved by the Food and Drug Administration for the treatment of T-cell lymphoma patients. In addition, FK228, PXD101, PCI-24781, ITF2357, MGCD0103, MS-275, valproic acid (VPA) and LBH589 are being studied in malignancies as monotherapy or in combination with other anti-tumor drugs [15].

In this study, it was aimed to determine the changes in *CDH1* gene expression level after administration of DNMT enzyme inhibitor (DAC) and Histone acetyltransferase enzyme inhibitor (TSA) in prostate cancer cell lines (LNCaP, PC3 and DU145) cells.

Materials and Methods

Cell culture and WST-1 cytotoxicity assay

DU145, P3 and LNCaP cells were cultured in RPMI 1640 medium containing 10% FBS in an oven set at 37°C in an environment with 95% humidity and 5% CO2. DU145, P3 and LNCaP cells were exposed to increasing doses of DAC and TSA. dH2O was used as the solvent for DAC, etanol was used as the solvent for TSA. After incubation, cytotoxic effects were investigated for each dose and duration. The experiment was repeated by making 3 separate cultures for each concentration. DU145, P3 and LNCaP cells were seeded in 96-well cell culture dishes at 10^5 cells/well. Then, they were incubated in RPMI 1640 medium prepared with various concentrations of DAC and TSA respectively, for 48 hours at 37°C with 5% CO^2. At the end of the specified times, 10 µl of WST-1 solution (Roche, Germany; Cat. No: 001 644 807 001) was added to each well and the color change caused by the formazan product was determined after 2 hours in DU145, P3 and LNCaP cells with a spectrophotometer at 450 nm wavelength. Negative controls were used blindly. Cell viability % calculations were performed with Excel program.

Total RNA isolation and cDNA synthesis

Total RNA isolation was performed after 48 h from DU145, PC3 and LNCaP cells incubated related DAC, TSA and DAC+TSA applications according to the PureZole isolation kit protocol steps (Biorad, USA, Cat. No: 732-6890). RNA quantity and purity were determined by NanoDrop ND-1000 V.3.7. The cDNA was obtained by using the iScript Reverse Transcription Supermix cDNA synthesis kit (Biorad, USA, Cat. No: 1708841) from 1µg total RNA in accordance with the protocol. 5 minutes 25ºC priming, 20 minutes 46ºC reverse transcription, 1 minute 95ºC RT inactivation. The obtained cDNAs were stored in a deep freezer at -20^0C until used in Real-Time PCR analysis.

Real time PCR analysis

mRNA levels of *CDH1* gene expressed in DU145, PC3 and LNCaP cells were determined by Real-Time PCR method using RotorGeneQ (Hilden, Germany). Amplifications was performed in 10 µL total reaction volume using related cDNA, site-specific primers (CDH1-PPH00135F-200,Qiagen;GAPDH-F5'CATTGCCCTCAACGACCACTTT 3', GAPDH-R 5' GGTGGTCCAGGGGTCTTACTCC 3'; Oligomer, Ankara), iTaq Universal SYBR Green Supermix (Biorad, USA, Cat. No: 1725122) and nuclease free water. We used the following Real-Time PCR protocol for *CDH1* and *GAPDH*: 95°C for 30 seconds initial denaturation followed by 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds;Melting curve analysis was performed for confirmation of single product amplification at the end of the PCR. 65-95°C, 0,5°C increments at 5 sec/step. Each run has been performed triplicate.

Statistical analysis

There are some free available software packages support statistical analysis of expression result. REST 2009 V2.0.13 and SPSS v.19 Software [16] were used for assessing the relative expression results.

Results

Evaluation of cell viability results

The effect of the DAC on cell viability after 48 hours incubation of DU145, PC3 and LNCaP cells is shown in figures 1, 2 and 3, respectively. Accordingly, it was determined that DU145, PC3 and LNCaP cells proliferation was decreased depending on dose and time. According to our results, the IC50 dose of DAC was determined 2 µM for DU145, 2 µM for PC3 and 1.8 µM for LNCaP in 48 hours of applications.

The effect of the histone deacetylase inhibitor TSA on cell viability after 48 hours incubation of DU145, PC3 and LNCaP cells

Figure 1: Cell viability rates determined after 48 hours of incubation of DU145 cells (5,000 cells/well) with DAC.*, IC50 value.

Figure 2: Cell viability rates determined after 48 hours of incubation of PC3 cells (5,000 cells/well) with DAC.*, IC50 value.

Figure 3: Cell viability rates determined after 48 hours of incubation of LNCaP cells (5,000 cells/well) with DAC.*, IC50 value.

is shown in figures 4, 5 and 6, respectively. Accordingly, it was determined that DU145, PC3 and LNCaP cells proliferation was decreased depending on dose and time. According to our results, the IC50 dose of TSA was determined 300 nM for DU145, 500 nM for PC3 and 200 nM for LNCaP in 48 hours of applications.

Figure 4: Cell viability rates determined after 48 hours of incubation of DU145 cells (5,000 cells/well) with TSA.*, IC50 value.

Figure 5: Cell viability rates determined after 48 hours of incubation of PC3 cells (5,000 cells/well) with TSA.*, IC50 value.

Figure 6: Cell viability rates determined after 48 hours of incubation of LNCaP cells (5,000 cells/well) with TSA.*, IC50 value.

Evaluation of gene expression levels

Rotor Gene Q was used for the quantitative evaluation of the expression level of the *CDH1* gene. GAPDH (Glyceraldehyde-3-Phosphate Dehydrogenase) gene was used for the normalization. The amplification curve of the Real-Time PCR reaction, which quantitatively shows the expression of *CDH1* and *GAPDH* genes at the mRNA level are shown in figure 7 and melt curves are shown in figure 8.

Figure 7: Amplification curves quantitatively showing the expression of the *CDH1* and *GAPDH* genes at the mRNA level. The Ct (Treshold Cycle) values of the Real Time PCR reaction of *CDH1* and *GAPDH* gene are located on the horizontal axis. Fluorescent signal is observed on the vertical axis.

Figure 8: Example of *CDH1* and *GAPDH* melting curve.

Dose determination of DNMT inhibitor DAC

Changes in mRNA level of *CDH1* gene after incubation of DU145, PC3 and LNCaP cells with 1, 1.4, 1.6, 1.8 and 2μM DAC for 48 hours are shown in figure 9, 10 and 11 respectively. The mRNA level of the *CDH1* gene in DU145 cells is increased 1.48-fold at 1 μM DAC, 1.36-fold at 1.4 μM DAC, 1.62-fold at 1.6 μM DAC, 1.59-fold at 1.8 μM DAC, and 2,73-fold at 2 μM DAC compared to control. Upregulation at 2 μM DAC was statistically significant ($p < 0.001$). The mRNA level of the *CDH1* gene in PC3 cells was increased 1.78-fold at 1 μM DAC, 2.77-fold at 1.4 μM DAC, 3.29-fold at 1.6 μM DAC, 4.82-fold at 1.8 μM DAC and 5,68-fold at 2 μM DAC compared to control. Upregulation in 1.4-2 μM DAC application was found statistically significant ($p < 0.001$). The mRNA level of the *CDH1* gene in LNCaP cells was increased 2.66-fold at 1 μM DAC, 2.36-fold at 1.4 μM DAC, 2.83-fold at 1.6 μM DAC, 6.82-fold at 1.8 μM DAC, and 3,2-fold at 2 μM DAC compared to control. Upregulations in all concentration applications were found statistically significant ($p<0.001$). The highest increase in *CDH1* expression level was detected at 2 μM DAC concentration in DU145 and PC3 cells, and at 1.8 μM DAC concentration in LNCaP cells.

Figure 9: Changes in *CDH1* mRNA level after 48 h of incubation of DU145 cells with 1 μM, 1.4 μM, 1.6 μM, 1.8 μM and 2 μM DAC. Expression levels of target genes were normalized based on *GAPDH* mRNA expression level,* $P < 0.001$.

Figure 10: Changes in *CDH1* mRNA level after 48 h of incubation of PC3 cells with 1 μM, 1.4 μM, 1.6 μM, 1.8 μM and 2 μM DAC. Expression levels of target genes were normalized based on *GAPDH* mRNA expression level,* $P < 0.001$.

Figure 11: Changes in *CDH1* mRNA level after 48 h of incubation of LNCaP cells with 1 µM, 1.4 µM, 1.6 µM, 1.8 µM and 2 µM DAC. Expression levels of target genes were normalized based on *GAPDH* mRNA expression level,* P < 0.001.

Figure 13: Changes in *CDH1* mRNA level after incubation of PC3 cells with 48h 2 µM DAC, 500 nm TSA and 42h DAC + 6h 500n m TSA. Expression levels of target genes were normalized based on *GAPDH* mRNA expression level,* P < 0.001.

DAC, TSA and DAC+TSA applications

When the changes in *CDH1* mRNA level were examined after incubation of DU145 cells with 2 µM DAC, 300 nm TSA and 42h DAC + 6h 300 nm TSA for 48 h, gene expression was upregulated compared to control in all three treatments (P < 0.001) (Figure 12). When the changes in *CDH1* mRNA level were examined after incubation of PC3 cells with 48 h of 2 µM DAC; 500 nm TSA and 42h of DAC + 6 h of 500 nm TSA; there was upregulation in 2 µM DAC application. Contrary to this, significant downregulation was detected in 500 nm TSA and 42h DAC + 6h 500nm TSA applications (P < 0.001) (Figure 13). When the changes in *CDH1* mRNA level were examined after incubation of LNCaP cells with 48 h 1.8 µM DAC, 200 nm TSA and 42h DAC + 6 h 200 nm TSA, significant upregulation was detected in 1.8 µM DAC application, while significant downregulation was detected in 200 nm TSA and 42h DAC + 6 h 200 nm TSA applications (P < 0.001) (Figure 14).

Figure 14: Changes in *CDH1* mRNA level after incubation of LNCaP cells with 48h 1.8 µM DAC, 200nmTSA and 42h DAC + 6h 200nm TSA. Expression levels of target genes were normalized based on *GAPD*H mRNA expression level,* P < 0.001.

Figure 12: Changes in *CDH1* mRNA level after incubation of DU145 cells with 2 µM DAC for 48 h, 300 nm TSA and 42h DAC + 6 h with 300 nm TSA. Expression levels of target genes were normalized based on *GAPDH* mRNA expression level,* P < 0.001.

Discussion

DNA methylation and other epigenetic mechanisms such as histone modifications act together to regulate gene expression through changes in chromatin structure [2,17]. Transcriptional suppression occurs through various mechanisms, such as aberrant methylation of CpG islands in the promoter, removal of proteins that prevent transcriptional factors from binding to the promoter, interacting with histone deacetylases, and, consequently, regulation of tumor phenotype. As a result, much of the methylation in a tumor may reflect its biological and clinical behavior [2,18]. Similarly, post-translational changes of histones are also associated with transcription regulation. During gene activation or suppression, both positive (H3Ac; H3K4me2; H3K4me3; H3K9Ac;

H4Ac) and negative acting markers (H3K9me3; H3K27me3) are determined between promoters, and the interaction of these histone modifications ultimately controls gene expression [19]. Importantly, the integration of environmental and intrinsic stimuli into gene expression control, as well as the interaction between DNA methylation and histone modifications during gene silencing. Abnormal promoter methylation has been studied in many genes in many cancer models by suppressing the gene [20-22].

In this study, we found that expression of *CDH1*, was inversely proportional to promoter methylation levels. It cannot be generally conclusively argued that increased expressions of the *CDH1* gene in cell lines after exposure to epigenetic modulating drugs capable of reversing DNA methylation and histone deacetylation are associated with a decrease in methylation levels. However, at the same time, it can be suggested that the high expression levels observed in our study when HDAC inhibitor was applied compared to the control were related to the demethylation agent in the study. These results suggested that histone modifications are most likely to be the main cause of *CHD1* silencing in cell lines. It may be suggested that the concentration of DAC to which the cell lines are exposed is sufficient to induce *CDH1* demethylation. However, only the same concentration is not effective to induce demethylation of the *CDH1* gene in different cell lines [23,24].

At the same time, using only TSA increased *CDH1* expression in DU145 cell line, while decreased it in PC3 and LNCaP cell line. However, *CDH1* expression was significantly upregulated in DU145 cells following exposure to both epigenetic modulating drugs. Whether the analyzed region of the *CDH1* gene is critical for regulation of expression can be evaluated. Alternatively, there is the possibility that exposure to DAC and TSA leads to reactivation of genes that positively regulate *CDH1*.

Differences in the frequency of hypermethylation of the *CDH1* promoter have also been demonstrated in studies between intestinal and diffuse gastric carcinomas [25-27]. In addition, Machado., *et al*. (2001) detected loss of expression in 9 gastric carcinoma samples with *CDH1* mutation and *CDH1* hypermethylation in six of them [25]. Tamura., *et al*. (2000) examined the protein expressions of e-cadherin and determined a loss or significant decrease in e-cadherin expression in 12 gastric carcinomas [26]. Cell-type-specific gene expression patterns are created and maintained through a complex of transcription factors and epigenetic regulators. In particular, DNA and histone modifications control the regulation of gene expression by controlling chromatin formation, structure and dynamics [2,28,29].

Conclusion

In conclusion, prostate cancer cell lines may be an ideal environment for testing demethylation drugs, but whether *CDH1* promoter methylation is a specific mechanism for e-cadherin suppression is uncertain. Therefore, failure of clinical activity of demethylating agents can be observed in the presence of methylated genes in some cases.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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