RESEARCH ARTICLE

Effect of Trichostatin A on CNE2 Nasopharyngeal Carcinoma Cells - Genome-wide DNA Methylation Alteration

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Abstract

Trichostatin A (TSA) is a histone deacetylase (HDAC) inhibitor. We here investigated its effects on proliferation and apoptosis of the CNE2 carcinoma cell line, and attempted to establish genome-wide DNA methylation alteration due to differentially histone acetylation status. After cells were treated by TSA, the inhibitory rate of cell proliferation was examined with a CCK8 kit, and cell apoptosis was determined by flow cytometry. Compared to control, TSA inhibited CNE2 cell growth and induced apoptosis. Furthermore, TSA was found to induce genome-wide methylation alteration as assessed by genome-wide methylation array. Overall DNA methylation level of cells treated with TSA was higher than in controls. Function and pathway analysis revealed that many genes with methylation alteration were involved in key biological roles, such as apoptosis and cell proliferation. Three genes (DAP3, HSPB1 and CLDN) were independently confirmed by quantitative real-time PCR. Finally, we conclude that TSA inhibits CNE2 cell growth and induces apoptosis in vitro involving genome-wide DNA methylation alteration, so that it has promising application prospects in treatment of NPC in vivo. Although many unreported hypermethylated/hypomethylated genes should be further analyzed and validated, the pointers to new biomarkers and therapeutic strategies in the treatment of NPC should be stressed.

Keywords: Trichostatin A - nasopharyngeal carcinoma - CNE2 – methylation - acetylation

Asian Pac J Cancer Prev, 15 (11), 4663-4670

Introduction

Nasopharyngeal carcinoma (NPC) is a rare malignant tumor in most parts of the world, but much more endemic in Southern China and Southeast Asia (Wue et al., 2010). The incidence of nasopharyngeal carcinoma is highest in those regions where the annual incidence is as high as 80 per 100,000 individuals (Spano et al., 2003). Currently, radiotherapy remains the mainstay of therapy for NPC, but the therapeutic effect is not always satisfactory (Qin et al., 2008). For patients with advanced disease, concomitant chemotherapy shows better therapeutic efficacy than radiotherapy given alone (Chan et al., 2005; Jamshed et al., 2014). Even with combined treatment, approximately 30% of patients experience treatment failure within 5 years, and treatment outcomes vary widely (Lee et al., 2010; Liu et al., 2013). It is urgent to find out more efficient therapeutic strategies and new biomarkers.

Tumors are characterized by aberrant transcriptional silencing of genes via epigenetic mechanisms, such as DNA methylation and post-translational modifications of the histones (Eriksson et al., 2013). DNA Methylation occurs mainly in the context of cytosines followed by guanines (CpGs) (Laurent et al., 2010). The methylation process is controlled by a family of DNA methyltransferases (DNMTs) that catalyze the transfer of methyl groups from S-adenosyl-L-methionine to the 5'-position of cytosine bases in the CpG dinucleotide (Fabbri et al., 2007). Altered DNA methylation, such as global hypomethylation or regional hypermethylation, is one of the most consistent epigenetic changes in human cancer (Gao et al., 2013). As for histones, transcriptional repression is in general associated with low levels of histone acetylation, whereas transcriptionally active genes are associated with highly acetylated core histones (Johnstone and Licht, 2003). Acetylation is believed to separate the basic N-termini of histones from DNA that then becomes more accessible to transcription factors (Cecconi et al., 2003). Thus, histone acetylation leads to gene activation, while histone deacetylation leads to a tighter histone-DNA interaction and, accordingly, to gene repression (Cecconi et al., 2003). Histone acetylation status is regulated by the opposing actions of histone acetyl-transferases (HATs) and histone deacetylases (HDACs) (Smith and Denu, 2009). During the last years HDAC-inhibitors have become one of the most promising classes of chemotherapeutics with profound antitumour effects (Glaser, 2007), and are successfully used to inhibit cell growth of lots of

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CNE2 cells proliferation was measured by Cell Counting Kit-8 detection kit (Beyotime, China). Cells were seeded into a 96-well plate at 5 × 10^4 cells per well with RPMI 1640 containing 10% FBS, and allowed to adhere for 24h. After the medium was removed, cells were washed with PBS, replace the previous medium with serum-free medium. Various concentrations of TSA (0, 100, 200, 300, 400, 500, 600 nM) were added to each well, respectively. At 6, 12, 24 and 36 h after incubation, CCK-8 solution was applied at 10 μl per well and followed by 2-h incubation at 37 °C. Absorbance values of all wells were then determined at 450 nm in Microplate Reader (Bio-Rad, USA). All experiments were conducted in triplicate.

**FCM analysis**

Annexin-V/propidium iodide (PI) double staining assay was performed by using the Annexin V-FITC apoptosis detection kit (Sigma-aldrich, USA) as described by the manufacturer’s instruction. Briefly, 1 × 10^5 cells per well with various treatments described for 24h were harvested and washed with cold PBS in 24-well plates. The cells were washed with PBS twice and resuspended in 1× Binding buffer, followed by incubated with Annexin V-FITC conjugate and PI for 15 min to protect from light at room temperature. Cells were analyzed by flow cytometry (FCM) analysis using BD CELLQuest software (BD Biosciences, USA).

**DNA extraction and sodium bisulfite conversion**

DNA extraction was performed on CNE2 cells treated with 0nm and 400nm TSA for 24h using the QIAamp DNA Mini Kit (Germantown, MD, USA). Bisulfite conversion of genomic DNA was done with the EZ DNA methylation Kit (Zymo Research, D5002) by following manufacturer’s protocol.

**Microarray**

Bisulfite-converted genomic DNA was analyzed using Illumina’s Infinium Human Methylation27 Beadchip Kit (Illumina Inc., USA). Beadchip contains 27,578 CpG loci covering more than 14,000 human RefSeq genes at single-nucleotide resolution. Chip process were performed by using reagents provided in the kit and following manufacturer’s manual. Data were extracted and summarized using genomeStudio software. Arrays that did not pass quality control in terms of b-distributions and expected p-values across the arrays were removed. Methylation scores represented as β values were generated for each site using Illumina Genome Studio Methylhation module v1.8 (Illumina Inc., USA) and were computed based on the ratio of methylated to methylated plus unmethylated signal outputs.

**Data-dependent bioinformatics for network analysis**

Functional classification of identified proteins was accomplished by using Entrez Gene (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db_gene) and DAVID (http://www.david.abcc.ncifcrf.gov). Proteins with multiple functions were assigned to those that are best known. Pathway module analysis of the functional clusters was performed by KEGG.
RT-QPCR analysis

The mRNAs were isolated from CNE2 cells using Trizol reagent from Invitrogen. Up to 1 μg of RNA was reversed-transcribed using the PrimeScript RT reagent Kit with gDNA Eraser (Takara, Japan). cDNA was amplified in SYBR green master mix (Roche, Germany) with the Realplex (Eppendorf, Germany). Conditions used for amplification were as follows: an initial 5 minute 95°C period followed by 40 cycles of 95°C for 15 seconds, 60°C for 1 min. Relative mRNA levels were determined using the \(2^{-\Delta\Delta CT}\) interpretation and normalized to the β-actin. The primer pairs for each gene were designed with Primer Premier 5.0 software; Sequences of all the primers are shown in Table 1.

Statistical analysis

All experiments were performed independently three times, the results were shown as mean values ± standard deviation (SD), and statistical analyses were performed using SPSS17.0 software. The statistical differences were calculated using a standard one-way ANOVA and two-tailed unpaired Student’s t test. \(P < 0.05\) was considered as statistically significant. In all graphs, *, # indicates significant difference.

Results

The cell proliferation inhibition effects of TSA

The cell proliferation inhibition effects of TSA in CNE2 cells were detected by CCK8 assay. The CNE2 cells were treated with 0, 100, 200, 300, 400, 500, 600 nM TSA respectively for 24, 48 and 72 h respectively. As shown in Figure 1, the TSA inhibited the proliferation of CNE2 cells was reduced in a time- and dose-dependent manner. From 300 nM to 500 nM, the inhibitory rate increased quickly. Some changes of apoptosis could be seen by light microscopy, such as cytoplasmic vacuoles and apoptotic bodies (Figure 1B). About 48h, most of the cells were dead (Data not shown).

The cell cycle arrest and apoptosis induced by TSA

Annexin V-FITC/PI double staining and FCM analysis were performed to evaluate the ability of TSA. As Figure 2 shows, the apoptosis of cells treated with TSA for 24h significantly increased compared with that of untreated cells (\(p<0.05\)). The inhibitory rate was highest in the cells treated with 400 nM TSA than that in other groups.

Global Methylation Profile

Global DNA methylation profiles were measured using Illumina Infinium Human Methylation27 BeadChips, which target 14, 475 total refseq genes, 12, 833 well-annotated genes described in the NCBI CCDS database, 144 methylation hotspots in cancer genes, 982 cancer-related targets and 110 miRNA promoters. We performed genome-wide methylation profiling between cells treated with TSA for 24h and control cells (0 nm, 24h). Preliminary analysis was conducted after background correction, then sites on the sex chromosomes were removed from the analysis on the array. 26468 CpG sites obtained. The average methylation intense of cells treated with 400nM TSA was lower than that of matched control (Figure 3A and Figure 3B), but they had the familiar frequency distribution (Figure 3B). Furthermore, most of the sites had positive correlation between TSA

Table 1. Sequences of the Primers for RT-QPCR

<table>
<thead>
<tr>
<th>Name</th>
<th>Strand</th>
<th>Sequence (5’–3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSPB1</td>
<td>Forward</td>
<td>AAGGATGGCGTGTTGGAGA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AAAAGGAGCAGGGAGGAAAA</td>
</tr>
<tr>
<td>DAP3</td>
<td>Forward</td>
<td>GGGTAATTGCTACCGAGG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GAGGCGAAGCCAGAGGAG</td>
</tr>
<tr>
<td>CLDN</td>
<td>Forward</td>
<td>GGGAGCGCAAAAGATGAGG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CTGCCAGGCCTAATAAGAG</td>
</tr>
<tr>
<td>NDFIP1</td>
<td>Forward</td>
<td>CTCCACCATATACGACGACAGT</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AGTAGCTTCAGCTGCTTGA</td>
</tr>
<tr>
<td>GRK5</td>
<td>Forward</td>
<td>AGAGCTCCTTCAGGCGCAG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GGTTTACGTCGACGAGGATG</td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward</td>
<td>TGGAGCACTTCTTCCAGCCT</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CCGATCCACACCGAGTACTTG</td>
</tr>
</tbody>
</table>

Table 2. Characteristics of Genes Selected to Verify

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Name</th>
<th>Gene ID</th>
<th>Strand</th>
<th>TSS</th>
<th>CPG Island</th>
<th>CPG Island Locations</th>
<th>Methylation</th>
</tr>
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<tbody>
<tr>
<td>HSPB1</td>
<td>heat shock 27kd protein 1</td>
<td>3315</td>
<td>Forward</td>
<td>9</td>
<td>TRUE</td>
<td>7:75769511-75771539</td>
<td>Up</td>
</tr>
<tr>
<td>CLDN7</td>
<td>Clostridium perfringens enterotoxin receptor-like 2</td>
<td>1366</td>
<td>Reverse</td>
<td>120</td>
<td>TRUE</td>
<td>17:7104851-710719</td>
<td>Up</td>
</tr>
<tr>
<td>DAP3</td>
<td>Death Associated Protein 3</td>
<td>7818</td>
<td>Forward</td>
<td>1422</td>
<td>TRUE</td>
<td>1:153924032-153926450</td>
<td>Up</td>
</tr>
<tr>
<td>NDFIP1</td>
<td>Nedd4 Family Interacting Protein 1</td>
<td>80762</td>
<td>Forward</td>
<td>287</td>
<td>TRUE</td>
<td>5:141468002-141468924</td>
<td>Down</td>
</tr>
<tr>
<td>GRK5</td>
<td>G protein-coupled receptor kinase 5</td>
<td>2869</td>
<td>Forward</td>
<td>354</td>
<td>TRUE</td>
<td>10:120955944-120958030</td>
<td>Down</td>
</tr>
</tbody>
</table>

TSS, Distance to transcription start site; Up, Hypermethylation; Down, Hypomethylation; All methylation locis are before TSS
treated cells and untreated cells, just a few not (Figure 3C). The comparison of methylation between groups was based on the difference in mean beta value (Avgβ) of each CpG site. Empirically, we selected the sites that the ratio between the Avgβ of cells treated with 400nM TSA and Avgβ of control cells is >1.5 or <0.67 as a threshold and \( p \)-value less than 0.05. There were 512 CpG sites that significantly differed in methylation level between the cells treated with TSA for 24h and control (0nm, 24h). Among all significant CpG sites, 97% were significantly hypomethylated (covering 488 genes) and 3% were significantly hypermethylated (covering 17 genes) in the cells treated with TSA, compared to control (Figure 3D).

Functional categories of the identified hypermethylated and hypomethylated gene associated with TSA

By searching Entrez Gene (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db_gene) and DAVID (http://www.david.abcc.ncifcrf.gov), we found most of the hypermethylated and hypomethylated gene associated with TSA treatment identified by methylation array were clustered in some functional categories. As shown in Figure 4A, these functional categories include nucleoside/nucleotide and nucleic acid metabolism, regulation of transcription, DNA-dependent, response to stress, apoptosis/regulation of apoptosis, cell proliferation/regulation of cell proliferation, regulation of developmental process, response to chemokine stimulus, regulation of transcription, DNA-dependent, response to stress, apoptosis/regulation of apoptosis, cell proliferation/regulation of cell proliferation, regulation of developmental process, response to chemokine stimulus.
neurotrophin signaling pathway, adherens junction, etc. Since drugs inducing apoptosis remain the main chemotherapeutic agents in medical oncology, we focus on the genes associated with apoptosis and cell proliferation. At the meantime, many genes did not report that their methylation alteration was related to NPC tumorigenesis.

The change of gene Expression after TSA treatment

The mRNA expression of DAP3, HSPB1, CLDN, NDFIP1 and GRK5 was detected by RT-qPCR (Table 2). mRNA expression of NDFIP1 and GRK5 did not change significantly. DAP3, HSPB1 and CLDN exhibited a significant decrease in the cells treated by TSA (400nM and 500nM for 24h compared with the control cells (Figure 5).

Discussion

Recent studies revealed that TSA, a HDAC inhibitor, has significant antitumor activity in many types of cancer cells. Expression levels of many genes are altered by TSA due to global suppression of histone deacetylation. In the present study, effects of TSA on NPC were investigated. Furthermore, genome-wide DNA methylation alteration regulated by TSA also been involved.

Accumulation of genetic and epigenetic alterations results in the development of NPC. As a main epigenetic change, histone hyperacetylation is generally associated with chromatin decondensation, which increases the accessibility of DNA to binding proteins as well as transcriptional activity, whereas histone hypoacetylation contributes to chromatin condensation and transcriptional repression (Tse et al., 1998; Wang et al., 2001). The interplay between HDACs and HATs differentially and reversibly regulates the acetylation status of the N-terminal region of histones as well as non-histone proteins (Johnstone and Licht, 2003; Marks et al., 2004; Dokmanovic et al., 2007). Transcriptional activators recruit HATs and are associated with acetylated chromatin (Gregory et al., 2001; Eberharter and Becker, 2002). In contrast, transcriptional repressors and co-repressors interact with HDACs and their binding to promoters correlates with loss of histone acetylation (Villar-Garea and Esteller, 2004; Saha and Pahan, 2006). Thus, HDACs function in opposition to HATs by removal of charge neutralizing acetyl groups from the histone lysine tails thereby inducing transcriptional repression through chromatin condensation (Roth et al., 2001; Thiagalingam et al., 2003). Aberrant HDAC expression and activity has been observed in numerous malignancies. These provided the basis for the development of HDAC inhibitors as anticancer therapies. It is possible to interfere with lysine acetylation by a pharmacological approach using HDAC-inhibitor. The final outcome of HDAC-inhibitor treatment is broad and cell type dependent (Shiva and Willems, 2014).

In our experiment, we found that TSA could induce CNE2 cells to death (Figure 1A), and this cell death included apoptotic cell death (Figure 2). At present, drugs inducing apoptosis remain the main chemotherapeutic agents in medical oncology (Lefranc and Kiss, 2006). Although cisplatin-based chemotherapy has shown promising effects with NPC, acquired resistance to chemotherapy has been one of the most important clinical problems (Kartalou and Essigmann, 2001; Siddik, 2003). Therefore, it is still an urgent need to explore new therapeutic strategies.

DNA methylation is another important epigenetic mechanism of regulating gene expression. In general, promoter DNA methylation is correlated with gene repression. When a gene is hypermethylated, especially in its promoter region, transcription is usually diminished (Vailant and Paszkowski, 2007). Duan X et al. reported that Abrant hypermethylation of H0XA2 led to low RNA expression in NPC tumors and cells (Li et al., 2013). Tian F, et al. found five hypermethylated genes (RASSF1, CDKN2A, DLEC1, DAPK1 and UCHL1) using methylation-specific PCR, and got a conclusion that Screening DNA hypermethylation of tumor suppressor genes in serum was a promising approach for the diagnosis of NPC (Tian et al., 2013). Du C, et al. posed Arsenic trioxide could reverse LMP1-mediated methylation and silencing of E-cadherin gene in NPC, and restore the gene’s promoter activity and expression, and indicate a potential therapeutic strategy for NPC (Du C et al., 2012).

Oncogenes could be silenced by inducing methylation of their promoter regions. Some researchers have postulated that reversal of both promoter methylation and histone deacetylation may lead to a greater degree of gene transcription than the reversal of one mechanism alone (Gilbert et al., 2004). An importance of epigenetic aberrations, including promoter hypermethylation and histone modifications, has been indicated for the processes of carcinogenesis and pathogenesis of Cholangiocarcinoma (Limaplaiboon, 2012). Furthermore, it has been found DNA methylation and histone modification regulate silencing of OPG during NPC progression (Lu et al., 2009). Although TSA is being evaluated in preclinical cancer models and in clinical trials, little is known about its mechanism of action. Since both DNA methylation and histone modifications are strongly implicated in the process of gene transcriptional activity, their relationship is a heated point (Ma et al., 2011). However, there is no consensus on which epigenetic mechanism initiates and steers this communication (Ma et al., 2011). Some experiments supported that DNA methylation guide histone modifications (Irvine et al., 2002; Locke and Martiensen, 2006; Huettel et al., 2007; Wozniak et al., 2007), however, other studies show contradictory evidence. Some researches demonstrated that increase in histone acetylation by Trichostatin A (TSA) was associated with a significant decrease in global methylation (Ou et al., 2007; Arzenani et al., 2011). In this study, we used a genome-wide DNA methylation microarray to measured global DNA methylation levels at 26468 CpG sites after TSA treatment. Overall DNA methylation levels of cells treated by TSA is lower than that in control. Furthermore, among 26468 CpG sites, we found 512 CpG sites to significantly differ in methylation level; 97% (512 loci) of which were hypomethylated (1.5 folder change).

Function and pathway analysis showed that most of genes of methylation alteration were clustered in diverse...
functional categories. As shown in Figure 4, TSA could affect many genes which had different functions, such as nucleic acid metabolism, transcription, immune, apoptosis, proliferation and ubiquitin. It indicated that TSA has diverse functions as other studies reported (Harrison and Dexter, 2013; Sigalotti et al., 2013). We focus on the genes associated with vital biological function, and did not report that their methylation alteration was related to NPC tumorigenesis before. Thus, some genes associated with apoptosis, proliferation and cell cycle were validated by RT-qPCR. We speculated that those genes may be involved in the PNC tumorigenesis.

HSPB1 coded protein is induced by environmental stress and developmental changes. It is involved in stress resistance and actin organization and translocates from the cytoplasm to the nucleus upon stress induction. In our experiments, this gene was hypermethylated, and mRNA expression was lower after TSA treatment. High level of HSPB1 expression in cancer cells promotes tumourigenesis, metastasis and anti-cancer drug resistance (Gibert et al., 2013). Wang JJ, et al. found up-regulated HSPB1 may play many important roles in the pathogenesis of colorectal cancer (Wang et al., 2012). HSPB1 also drives epithelial mesenchymal transition, metastasis, and circulating tumor cells in prostate cancer (Selker, 1998). It was identified as a radiosensitivity-related protein in NPC cells (Zhang et al., 2012). HSPB1 was thought as a new cancer therapeutic target (Arrigo and Gibert, 2012). DAP3 (death associated protein 3) is a GTP-binding constituent of the small subunit of the mitochondrial ribosome, and is responsible for regulating apoptosis induced by various stimuli (Harada et al., 2010). It is overexpressed in human thyroid oncocytes tumours, and may participate in mitochondrial maintenance and play a role in the balance between mitochondrial homeostasis and tumourigenesis (Jacques et al., 2009). CLDN (claudin 7) encodes a member of the claudin family. Claudins are integral membrane proteins and components of tight junction strands. Tight junction strands act as a physical barrier to prevent solutes and water from passing freely through the paracellular space between epithelial or endothelial cell sheets, and also play critical roles in maintaining cell polarity and signal transductions (Lu et al., 2013). Claudin-7 is frequently overexpressed in ovarian cancer and promotes invasion (Dahiya et al., 2011). Increased expression of CLDN contributes to an anti-apoptotic role in TNF-alpha-induced apoptosis (Liu et al., 2012). Uregulated CLDN expression confers resistance to cell death of nasopharyngeal carcinoma cells (Lee et al., 2010). Claudin 4 and claudin 7 may be a novel biomarker for the prediction of distant metastasis and unfavorable prognosis in NPC (Hsueh et al., 2010).

The mechanism that TSA effect the NPC cells are still quite complex, and the details and pathways involved should be clarified in the future. In conclusion, we first used genome-wide methylation arrays to identify genes methylated in CNE2 cells treated by TSA. Our study demonstrates that DNA methylation is involved in proliferation inhibitor and apoptosis induce by TSA. Although many novel methylated genes should be further analyzed and validated, it may still provide some new biomarkers and therapeutic strategies in the treatment of NPC.

Acknowledgements

The authors gratefully acknowledge the financial support provided by the Foundation for the Guangxi Key Laboratory for Prevention & Treatment of Regional High-Incidence Diseases (N0. KFJJ2010-35 ). We would like to thank Editage for providing editorial assistance.

References


