Downregulation of connexin 43 in nasopharyngeal carcinoma cells is related to promoter methylation

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Summary Down-regulation of Cx43 expression had been shown to occur in nasopharyngeal carcinoma cells. The present study was undertaken to estimate if methylation of the promoter region in Cx43 gene was responsible for the repression of Cx43 expression in the CNE-1 nasopharyngeal carcinoma cells. Calcein transfer and lucifer yellow transfer were detected to evaluate gap junction intercellular communication (GJIC) in CNE-1 cells. It was found that the control CNE-1 cells showed no fluorescent dye transfer. After treatment with DNA methyltransferase inhibitor 5-aza-CdR, fluorescent dye transfer between cells became obvious. RT-PCR and Western blot were performed to determine the expression of Cx43 gene. The control CNE-1 cells showed a low expression level of Cx43, whereas 5-aza-CdR-treated CNE-1 cells showed an enhanced level of Cx43 expression. Methylation-sensitive restriction enzyme and PCR analysis showed that the methylation of the Cx43 gene promoter region occurred in CNE-1 cells. In addition, treatment with 5-aza-CdR inhibited the growth (including anchorage-independent growth) of CNE-1 cells, and resulted in an accumulation of cells in G0/G1 phase. These results indicate the promoter methylation as an important role in inactivation of Cx43 in CNE-1 cells.

KEYWORDS
Connexin 43; Gap junction intercellular communication; Methylation; Nasopharyngeal carcinoma

Introduction

Nasopharyngeal carcinoma (NPC) is a type of head and neck cancer. It occurs sporadically in the west but is endemic in southern China and Southeast Asia. In the year 2000, a total of 64,798 new cases were registered worldwide, and more than 80% of those were reported from these areas. In southern China, nasopharyngeal carcinoma (NPC) is the third most common form of malignancy amongst men, with incidence rates of between 25 and 30 per 100,000 persons per year. Intercellular communication through gap junction (GJIC) plays a significant role in maintaining tissue homeostasis by exchanging small molecules, such as sugars, nucleotides, and second messengers, which has long been proposed as a mechanism to regulate growth control, development and differentiation. Dysfunctional GJIC has been also recognized as being involved in carcinogenesis. It has been showed that down-regulation of connexin 43
(Cx43) expression and dysfunctional GJIC occur in nasopharyngeal carcinomas tissues and cells, suggesting that dys-functional GJIC plays a key role in nasopharyngeal carcinogenesis.

The methylation within promoters is associated with transcriptional silencing of tissue-specific genes, X chromosome-linked gene inactivation, and some repetitive elements. It has been observed that normally unmethylated CpG islands can become aberrantly methylated or hypermethylated in neoplastic cells, and this has been suggested to be an important and common mechanism for transcriptional silencing of many tumor suppressor genes. Methylation has been reported to occur in the promoter region of Cx43 gene in various tumor cells, which is responsible for suppression of Cx43 expression and loss of Cx43-mediated GJIC. The present study was undertaken to estimate whether methylation of the promoter region in Cx43 gene was responsible for the repression of Cx43 expression in the CNE-1 nasopharyngeal carcinoma cells.

Materials and methods

Cell culture

CNE-1 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies Inc.) supplemented with 10% fetal bovine serum (FBS) (HyClone), 100 U/ml penicillin (Sigma) and 100 μg/ml streptomycin (Sigma) in a humidified atmosphere containing 5% CO2 at 37°C.

5-aza-2'-deoxycytidine Treatment

5-aza-2’-deoxycytidine (5-aza-CdR) was obtained from Sigma. The CNE-1 cells were plated at a density of 5 x 10^5 cells/ml. When the cells became confluent, 5-aza-CdR at the different final concentration (0.25 μM ~ 25 μM) was added to the medium. After treatment with 5-aza-CdR for 24 h, the cells were washed with phosphate-buffered saline (PBS) and fresh medium was added. Seventy-two hours later, the treated cells were used for the following assays.

Gap junction intercellular communication assays

Lucifer yellow and calcein transfer assays were used to measure GJIC in confluent CNE-1 cells treated with 5-aza-CdR. These techniques were carried out as follows:

Calcein transfer assay

GJIC was measured using calcein transfer assay as previously described. Briefly, for loading with calcein acetoxymethyl ester (calcein AM) (Fluka Sigma), donor cells were trypsinized with 0.25% trypsin solution, suspended in culture medium, centrifuged, re-suspended in 2 ml of staining solution (5 μM calcein AM, and 0.3 M glucose), and fluorescence-labeled for 30 min at 37°C. The cells were then washed with PBS three times, and added to a monolayer culture of unstained recipient cells. After incubation for 3 h, these co-cultures were trypsinized, suspended in PBS, and analyzed with a Becton Dickinson FACScan. The membrane-permeable calcein AM is hydrolyzed by intracellular nonspecific esterase and the resulting green fluorescent hydrophilic calcein is then trapped inside the cells. Therefore, gap junctions are permeable for calcein. In flow cytometry analysis, two peaks of calcein fluorescent density will appear in the obtained diagram. The right one represents the donor cells, and the left one means the recipient cells. If the cells demonstrate functional GJIC, the left peak will shift to right in co-cultured cells compared with the mixture of donor cells and recipient cells without incubation at 37°C.

Lucifer yellow transfer

GJIC was also measured by microinjection of the fluorescent dye Lucifer yellow CH (10% in 0.33 M LiCl) into selected cells by means of a micromanipulator and a micro-injector system (Olympus). Five minute after injection, the number of fluorescent cells around each injected single cell, which was loaded with the dye, was scored. Ten individual cells were injected per dish and means of number of fluorescent neighboring cells were calculated. Images were taken with an Olympus fluorescence microscope.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted using Trizol Total RNA MiniPreps Classic Kit (Sangon, Shanghai, China) as described in the manufacturer’s instructions. The RNA quantity was identified from the absorbance at 260 nm. First strand cDNA was synthesized using 25 μg total RNA by M-MuLV reverse transcriptase (Sangon, Shanghai, China) in a 100 μl reaction system.

PCR was carried out using 10 μl of cDNA in a 50 μl reaction with UNOII Thermocycler (Biometra). The sense primer for human Cx43 was 5’TGGACAGTCTGCCCTTGTGTTG-3’, and the antisense primer was 5’CCATACGTGGGGCACACCTTG-3’. The expected amplified fragment for Cx43 was 219 bp. As an internal control, the sense primer for β-actin gene was 5’TTGACCTTCCGCTAGAGTGG-3’, and the antisense primer was 5’ATCTCCTTCTGCATCATCGTG-3’. The expected amplified fragment for β-actin was 289 bp. The amplification reactions were initiated by a denaturation step for 4 min at 95 °C and then subjected to 28 cycles of 95 °C for 1 min, 60 °C for 45 s, and 72 °C for 45 s. The amplified DNA products were separated on 2% agarose gel, stained with ethidium bromide, visualized and photographed with ImageMaster Video Documentation System (Pharmacia Biotech).

Western blotting

Western blotting were performed to assay the protein level of Cx43. Briefly, the cells were lysed with 20% SDS containing 1 mM phenylmethylsulfonyl fluoride (Sigma), 1 mM leupeptin (Sigma), 1 mM antipain (Sigma), 0.1 M aprotinin (Sigma), 0.1 mM sodium orthovanadate (Sigma) and 5 mM sodium fluoride. After sonication the lysates, protein concentrations were determined using the BCA protein assay kit (Beyotime Biotechnology). Proteins were separated on...
12.5% SDS polyacrylamide gels and transferred to PVDF membranes (Pall Gelman Laboratory). The separated proteins were reacted sequentially for 1 h at room temperature with primary antibodies for Cx43 (Zymed) and β-actin (Sigma), followed by fluorescein-conjugated secondary antibody, detected with ECF chemifluorescence Western blotting Kit (Amersham Biosciences), visualized and obtained photos with a Storm 860 PhosphorImager system (Amersham Pharmacia Biotech).

Methylation-sensitive restriction enzymes and PCR

Genomic DNA was isolated from CNE-1 cells by a Classic Genomic DNA Isolation Kit (Sangon, Shanghai, China) as described in the manufacturer’s manual. Methylation status of promoter region was determined by digestion with methylation-sensitive-restriction enzyme, followed by a PCR method. Briefly, genomic DNA was digested completely with restriction endonucleases EcoRV, methylation-insensitiveMspI, methylation-sensitive HpaII or methylation-sensitiveHhaI (Sangon, Shanghai, China). For the double digestion, DNA was digested overnight with EcoRV (6 U/μg) at 37 °C, and then a part of the EcoRV-digested DNA was subsequently digested overnight with MspI (6 U/μg), HpaII (6 U/μg) or HhaI (6 U/μg) at 37 °C. To determine the target fragment (−1512 to −1233) of promoter region in human Cx43 gene, in which there are 12 CpG dinucleotides, 1 MspI/HpaII site and 2 HhaI sites, 100 ng of digested DNA was amplified by PCR. The sense primer for the target fragment was 5’-TGCAACATTGGGCCCAG-3’, and the antisense primer was 5’-TCTGTTAGACCGTTC-3’. PCR was performed with at first an initial denaturation at 95 °C for 4 min, followed with 35 cycles of 95 °C for 1 min, 62 °C for 1 min, and 72 °C for 2 min, and a final extension for 72 °C for 7 min. The amplified DNA products were separated on 2% agarose gel, stained with ethidium bromide, visualized and photographed with ImageMaster Video Documentation System (Pharmacia Biotech). PCR products (280 bp) will be found at the promoter having methylated status when digested with HpaII or HhaI. No products will be found at the promoter having unmethylated status when digested with HpaII or HhaI. All of the samples digested with MspI will have no products. To rule out the possibility of incomplete digestion, all samples were digested twice with each of the enzymes in independent experiments. PCR amplifications from each of the duplicate digests were repeated at least twice to ensure reproducibility of the results.

Cell growth analysis

For measuring the effect of 5-aza-CdR on cell growth, CNE-1 cells were seeded and cultured in six-well plates at 1 × 10⁵ cells/well. When the cells were confluent, the medium was replaced with fresh medium containing 5-aza-CdR at different concentrations. After treatment with 5-aza-CdR for 24 h, the cells were washed with PBS and fresh medium was added. Seventy-two hours later, the cell number in each well was counted using a haemocytometer.

Anchorage-independent growth assay

In each plate, one hundred cells in 2.5 ml of 0.33% agarose medium were plated on the top of 2.5 ml pre-hardened 0.5% agarose medium. After incubation at 37 °C for 1 day, the medium containing 5-aza-CdR at different concentrations was added on the top of the agar, and then incubation continued. Ten days later, the plates were dried at 37 °C for 1 day and anchorage-independent colonies of cells were stained with 1 mg/ml of 2-(p-iodophenyl)-3-(nitrophenyl)-5-phenyltetrazolium chloride (Sigma). The number of colonies in each plate was counted. The cloning efficiency was defined as the number of colonies per one hundred plated cells. The values from three independent experiments were averaged to obtain the final cloning efficiency of anchorage independence.

Cell cycle analysis

Cells were collected by centrifugation, fixed by 70% ethanol and re-suspended at 1 × 10⁶ cells/ml in propidium iodide (PI) staining buffer (0.1% sodium citrate, 0.1% Triton-X 100, and 50 μg/ml PI) and were treated with 1 mg/ml RNase at room temperature for 30 min. Cell-cycle histograms were generated after analysis of PI-stained cells with a Becton Dickinson FACScan. For each culture, at least 1.5 × 10⁴ events were recorded. Histograms generated by FACS were analyzed by ModFit Cell Cycle Analysis Software V2.0 to determine the percentage of cells in each phase (G1, S, and G2/M).

Results

5-aza-CdR induces restoration of GJIC

5-aza-CdR is an inhibitor of DNA methyltransferase. In this study, we determined the changes of GJIC in CNE-1 cells after treatment with 5-aza-CdR. Fluorescent dye calcein transfer through gap junction was determined using flow cytometry to evaluate functional gap junctions (Fig. 1A).

As expected, in control CNE-1 cells, after co-culture of recipient cells and donor cells for 3 h, the recipient cell peak (the right one of black peaks) only slightly shifted to right compared with the recipient cell peak (the right one of gray peaks) of mixed cells without co-culture, which suggests the control CNE-1 cells showed no dye transfer and no functional gap junctions. After CNE-1 cells were treated with 5-aza-CdR at the concentration of 25 μM, the recipient cell peak obviously shifted to right, indicating that the treatment with 5-aza-CdR restored GJIC.

In order to further validate the effects of 5-aza-CdR up-regulating GJIC, fluorescent dye Lucifer yellow CH was injected into a single cell, and lucifer yellow transfer through gap junction was observed using fluorescence microscope (Fig. 1B). It was observed that the number of communicating cells per injected cell was 10.0 ± 1.0 cells in 5-aza-CdR-treated cells, while there was almost no communicating cell in control CNE-1 cells. These results indicate that 5-aza-CdR treatment can restore GJIC of CNE-1 cells.

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5-aza-CdR enhances the expression of Cx43

Cx43 protein is one type of general sub-unit of gap junction semi-channel. To evaluate the mechanism of GJIC restoration by 5-aza-CdR, it was detect whether treatment with 5-aza-CdR altered the expression of Cx43 gene. RT-PCR was performed to determine the mRNA level of Cx43 gene. We observed that the control CNE-1 cells showed low mRNA expression level of Cx43, whereas 5-aza-CdR-treated CNE-1 cells showed an enhanced level of Cx43 transcript (Fig. 2A). Protein expression of Cx43 was also analyzed by Western blot. As shown in Figure 2B, 5-aza-CdR up-regulated Cx43 protein levels. These results suggest that the silencing of Cx43 gene and deficiency in GJIC in CNE-1 cells might relate to DNA methylation.

Methylation status in the promoter region of Cx43 gene

In order to further clarify methylation of the Cx43 gene promoter region in CNE-1 cells, we used the methylation-sensitive restriction enzyme/PCR. A 280 bp DNA fragment is successfully amplified from EcoRV-digested genomic DNA from CNE-1 cells (Fig. 3). When EcoRV-digested genomic DNA was further digested with methylation-insensitive restriction enzyme Mspl, no PCR product was found. However, when EcoRV-digested genomic DNA was further digested with methylation-sensitive restriction enzyme HpaII or HhaI, the PCR products could be observed. These results indicate that the silencing of Cx43 gene expression
in CNE-1 cells correlates with methylation of the gene promoter region.

5-aza-CdR inhibits the growth of CNE-1 cells

We further determined the effects of increasing concentrations of 5-aza-CdR on cell growth. After confluent CNE-1 cells were treated with 5-aza-CdR for 24 h, the medium was replaced. Seventy-two hours later, the cell number in each well was counted. It was observed that the growth of CNE-1 cells was inhibited in a concentration-dependent manner (Fig. 4A). 5-aza-CdR at concentration of 25 \( \mu \text{M} \) caused over 50% decrease of cell growth but without obvious cytotoxicity.

Flow cytometry analysis was conducted to analyze the cell cycle of CNE-1 cells treated with 25 \( \mu \text{M} \) 5-aza-CdR. The treatment induced G0/G1-phase cell cycle arrest of CNE-1 cells concomitant with a decrease in S phases (Fig. 4C). The percentage of cells in G0/G1 phase in 5-aza-CdR-treated cells increased to 60.45% from 45.30% compared with the control cells, whereas the percentage of cells in S phase decreased to 22.82% from 37.46%, respectively. The number of cells in G2/M phase showed no difference.

The effect of 5-aza-CdR on anchorage-independent growth of CNE-1 cells in soft agar was also detected. After CNE-1 cells were plated in soft agar and cultured for 24 h, the medium containing 5-aza-CdR was added on the top of the agar. Ten days later, the colony formation in soft agar was assayed. Data represent the mean and SD from three independent experiments. * \( p < 0.01 \) vs. solvent control.

Discussion

Loss of GJIC has been linked to aberrant proliferation and an enhanced neoplastic phenotype. Many human tumors have been reported to be deficient in expression of the connexin (Cx) genes and GJIC, and Cx genes have been suggested as tumor suppressor genes.\(^3\)\(^4\) However, mutations in Cx genes

\[ \text{Maeker} \rightarrow \text{EcoRV} \rightarrow \text{EcoRV/MspI} \rightarrow \text{EcoRV/HpaII} \rightarrow \text{EcoRV/HhaI} \]

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\text{Figure 3} & \quad \text{Cx43 promoter methylation status analyzed with Methylation-sensitive restriction enzyme/PCR in CNE-1 cells. After genomic DNA was digested with EcoRV, the EcoRV-digested DNA was subsequently digested overnight with MspI, HpaII or HhaI. Then, PCR for Cx43 was carried out. EcoRV, DNA only digested with EcoRV. EcoRV/MspI, DNA digested with EcoRV and MspI. EcoRV/HpaII, DNA digested with EcoRV and HpaII. EcoRV/HhaI, DNA digested with EcoRV and HhaI.}
\end{align*} \]

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\text{Figure 4} & \quad \text{The Changes of cell growth and Cell cycle of CNE-1 cells after treatment with 5-aza-CdR. (A) Effects of 5-aza-CdR on growth of CNE-1 cells. After treatment with 5-aza-CdR at indicated concentrations for 24 h, the medium was replaced. Seventy-two hours later, the cell number in each well was counted. Data represent the mean and SD from three independent experiments. (B) The effect of 5-aza-CdR on anchorage-independent growth of CNE-1 cells. CNE-1 cells were plated in soft agar 100 cells pre plate. After 1 day, the medium containing 5-aza-CdR at indicated concentrations was added on the top of the agar. Ten days later, the colony formation in soft agar was assayed. Data represent the mean and SD from three independent experiments. * \( p < 0.01 \) vs. solvent control. (C) Cell cycle analysis of CNE-1 cells after treated with 5-aza-CdR. After the confluent CNE-1 cells were exposed to 5-aza-CdR at 25 \( \mu \text{M} \) for 24 h, the medium was refreshed. Seventy-two hours later, the cells were stained with propidium iodide, and 15,000 cells of each sample were analyzed under FACScan. Data represent the mean and SD from three independent experiments. * \( p < 0.01 \) vs. solvent control.}
\end{align*} \]
including Cx43 were found to be rare events in carcinogenesis. It is well established that promoter methylation can result in decreased expression of tumor suppressor genes such as p16, p53, RB, VHL, hMLH-1, APC and BRCA1 genes, and contributing to increased tumorigenicity in a variety of human cancers. Previous studies have shown that cell-specific transcription of Cx32 and Cx43 in hepatic cells is controlled by promoter methylation, suggesting that the methylation within promoters might involve in transcriptional inhibition of connexin genes in cancer cells. Downregulation of Cx43 expression and dysfunctional GJIC had been shown to occur in nasopharyngeal carcinoma tissues and cells. In our present study, treatment with 5-Aza-CdR induced the re-expression of Cx43 at mRNA and protein levels, and restored the capacity of cell-cell communication in CNE-1 cells. These results suggested that the silencing of Cx43 gene depended on the methylation of the promoter region in CNE-1 cells. In order to confirm this possibility, we estimated whether the methylation occurred in the promoter region of Cx43 gene in CNE-1 cells using methylation-sensitive restriction enzymes and PCR method. The results demonstrated that methylation of the Cx43 promoter region occurred in CNE-1 cells. This indicated that the methylation of the promoter region was an important factor to induce the repression of the Cx43 gene.

The promoter of the human Cx43 genes has been well cloned. Several functional cis-acting elements, including AP-1 and AP-2 response elements, Sp1 response element and estrogen response elements, have been described in the human Cx43 promoters. The AP-1 sites in the human Cx43 promoter function positively since phorbol ester treatment increased and loss of Cx43-mediated GJIC in HeLa cells. AP-1 sites can induce the repression of the Cx43 gene. The human Cx43 promoter contains several half-palindromic estrogen-responsive elements (EREs) that enhance Cx43 transcription in the presence of estrogen. Thus, we suggest that aberrant transcription factors binding with promoter region by methylation may be responsible for the aberrant Cx43 gene transcription.

The methylation at the promoter and proximal transcribed region has been reported to be responsible for the repression of Cx43 gene and other connexin genes in other various tumor cells. It has been reported that aberrant Cx43 mRNA expression was regulated by promoter methylation in non-small cell lung cancer, and this aberrant expression was correlated with nodal micrometastasis. King et al has shown that treatment of Cx43-negative clones from HeLa cells with methylation inhibitor 5-aza-2′-deoxycytidine resulted in expression of Cx43, suggesting that methylation of the Cx43 gene promoter, or a gene regulating this promoter, might be responsible for suppression of Cx43 expression and loss of Cx43-mediated GJIC in HeLa cells. Hypermethylation in the promoter region is a mechanism for the Cx32 gene repression in human renal cell carcinoma cells. Cx26 is a structural component of gap junctions expressed in breast epithelial cells. Expression levels of Cx26 are reduced in many breast tumors. Tan et al have reported that methylation of the promoter region of the Cx26 gene is likely to be an important mechanism in modulating the expression of Cx26 in breast cancer. Another report showed that downregulation of Cx26 in human lung cancer was also related to promoter methylation. These results suggested that promoter methylation might be a common mechanism for repression of connexin genes in various tumor cells. However, we cannot exclude the possibility that some other factors are involved in the repression of the Cx genes. A recent study has showed that methylation was probably not involved as a primary mechanism of Cx26 regulation in human esophageal cancer cell lines. It has been well revealed that another important mechanism that mediates the repression of gene transcription, either activation or repression, is the acetylation status of nuclear histones by histone acetyltransferases (HAT) and histone deacetylase (HDAC). In general, histone acetylation by HAT favors the open chromatin structure that is more transcriptionally active, while deacetylation by HDAC favors chromatin condensation that is prone for repression of gene transcription. Recently, a HDAC-dependent mechanism was reported to be responsible for the transcriptional repression of Cx43 in prostate cancer cells, which affect Sp1 and AP1 transcription factors together with the coactivator/adaptor p300/CBP and possibly other factors to regulate Cx43 gene transcription. In addition, extracellular matrix protein fibronectin and overexpression of estrogen receptor-alpha gene has been shown to suppress the expression of Cx26 in alveolar epithelial cells and endometrial carcinoma cells, respectively. Further studies are required to dissect whether these pathways regulate the expression of Cx genes in various cancer cells.

Cx43 and other connexin protein have been indicated to function as a tumor suppressor. Transfection of connexin 43 decreases neoplastic potential as evidenced by attenuated anchorage-independent growth. We further determined the effects of increasing concentrations of 5-aza-CdR on cell growth. Our results show that together with increasing gap junction communication and Cx43 expression, 5-aza-CdR significantly decreases the cell proliferation and anchorage-independent growth. The inhibition of cell proliferation and anchorage-independent growth is likely due to a block of cell cycle progression, because 5-aza-CdR increased the percentage of cells in the G0/G1 coinciding with a significant decrease in the number of cells in the S phase. In fact, previous studies have indicated that the re-establishment of gap junctions with the restoration of gap junctional communication affects the phenotype of transformed or tumor cells involved in cell cycle regulation. Transfection and overexpression of Cx43 genes reverted phenotypically transformed dog kidney epithelial cells to a flat morphology and restored the cells sensitive to density-dependent inhibition of proliferation with their G1- and S-phase duration almost doubled. Another study showed that overexpression of Cx43 suppressed proliferation of human osteosarcoma U2OS cells through inhibition of the cell cycle transition from G1 to S phase. Tolbutamide, a sulfonylurea compound, has been shown to enhance gap junction permeability in the poorly coupled C6 glioma cells with an increase of Cx43 expression. The increase in communication is concurrent with the inhibition of the rate of proliferation due to a block of the progression of C6 glioma cells through the S phase of the cell cycle. Overall, it seems that the down-regulation of Cx43 gene mediated through the methylation of the promoter regions in nasopharyngeal carcinoma cells. Treatment of DNA meth-
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References