Pyrrrole-imidazole polyamide-mediated silencing of KCNQ1OT1 expression induces cell death in Wilms' tumor cells

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Received March 5, 2015; Accepted April 8, 2015

DOI: 10.3892/ijo.2015.3018

Abstract. KvDMR (an intronic CpG island within the KCNQ1 gene) is one of the imprinting control regions on human chromosome 11p15.5. Since KvDMR exists within the promoter region of KCNQ1OT1 (antisense transcript of KCNQ1), it is likely that genomic alterations of this region including deletion, paternal uniparental disomy and de-methylation in maternal allele lead to aberrant overexpression of KCNQ1OT1. Indeed, de-methylation of KvDMR accompanied by uncontrolled overexpression of KCNQ1OT1 occurs frequently in Beckwith-Wiedemann syndrome (BWS), and around 10% of BWS patients developed embryonal tumors (Wilms' tumor or hepatoblastoma). These observations strongly suggest that silencing of KCNQ1OT1 expression might suppress its oncogenic potential. In the present study, we designed two pyrrole-imidazole (PI) polyamides, termed PI-a and PI-b, which might have the ability to bind to CCAAT boxes of the KCNQ1OT1 promoter region, and investigated their possible antitumor effect on Wilms' tumor-derived G401 cells. Gel retardation assay demonstrated that PI-a and PI-b specifically bind to their target sequences. Microscopic observations showed the efficient nuclear access of these PI polyamides. Quantitative real-time PCR analysis revealed that the expression level of KCNQ1OT1 was significantly decreased when treated with PI-a and PI-b simultaneously but not with either PI-a or PI-b single treatment. Consistent with these results, the combination of PI-a and PI-b resulted in a significant reduction in viability of G401 cells in a dose-dependent manner. Furthermore, FACS analysis demonstrated that combinatory treatment with PI-a and PI-b induces cell death as compared with control cells. Taken together, our present observations strongly suggest that the combinatory treatment with PI polyamides targeting KCNQ1OT1 might be a novel therapeutic strategy to cure patients with tumors overexpressing KCNQ1OT1.

Introduction

Most autosome genes are normally expressed from both their paternal and maternal alleles. In contrast, imprinted genes are expressed predominantly and/or exclusively from either of their paternal or maternal allele (1). For example, certain imprinted genes such as IGFI2, DMT1 and PEG3 are expressed from their paternal alleles, while their maternal alleles are silenced. Conversely, others including RB1, TP73 and CDKNIC are expressed from their maternal alleles, whereas their paternal alleles are inactivated. Abnormal regulation of imprinting causes fetal death or development of diseases such as Beckwith-Wiedemann syndrome (BWS), Angelman syndrome and Prader-Willi syndrome.

BWS has been considered to be an overgrowth disorder accompanied by several symptoms including macrosomia, macroglossia and abdominal wall defects (2,3). In addition, patients with BWS have been shown to be predisposed to childhood and embryonal tumors such as Wilms' tumor and hepatoblastoma (4-7). Majority of patients with BWS harbor aberrant alterations at human chromosome 11p15 (3). Both epigenetic and genomic alterations of the imprinting cluster within this chromosomal region have been detected in up to 80% of BWS patients as examined by currently available
procedures (8,9). The imprinting of 11p15.5 region was found to be regulated by two imprinting control regions, H19DMR located upstream of the H19 gene and KvDMR within intron 10 of KCNQ1 gene, which acts as a promoter region of its antisense gene KCNQ1OT1 (10).

KCNQ1OT1 encodes a paternally expressed long non-coding RNA, which has been shown to regulate imprinting of several genes present at 11p15.5 locus in cis (11,12). Under normal conditions, the maternal and paternal alleles of KvDMR are methylated and unmethylated, respectively (10). Loss of imprinting of this region, i.e. loss of methylation of paternal allele, resulted in aberrant overexpression of KCNQ1OT1, which suppressed the circumspect gene expression such as the CDKNIC (p57KIP2) tumor suppressor gene (13,14).

p57KIP2 is a maternally expressed imprint gene encoding a cyclin-dependent protein kinase inhibitor implicated in the regulation of prenatal and postnatal development (15). Defects of imprinting at KvDMR account for the majority (~50%) of molecular aberrations in BWS patients, which causes the biallelic expression of KCNQ1OT1 and then leads to a significant suppression of p57KIP2 expression (16,17). Moreover, loss of methylation at KvDMR in association with reduced expression level of p57KIP2 was also observed in a variety of adult tumors including colorectal cancer and lung cancer (18,19). These findings indicate that silencing of KCNQ1OT1 might prohibit tumor development and/or progression.

Pyrrole-imidazole (PI) polyamide has been shown to be a novel gene-silencing small chemical compound, which binds to a minor groove of double-strand DNA in a sequence-specific manner (20). Hairpin polyamide is derived from distamycin A, which binds to AT-rich DNA sequences (21). Polyamide containing the appropriate combination of aromatic amino acids N-methylpyrrole (Py) and N-Methylimidazole (Im) binds to its target DNA with affinity and specificity comparable to that of DNA-protein interaction in cells (22). According to the previous observations, Im/Py, Py/Im and Py/Py pairs bind to G-C, C-G, and A-T/T-A, respectively (22).

In the present study, we have generated the PI polyamides targeting the CCAAT box, which is present at KvDMR and involved in the regulation of KCNQ1OT1 expression (10), and examined whether these compounds could specifically suppress growth of Wilms' tumor-derived G401 cells.

Materials and methods

Cell lines and culture conditions. Human fibroblasts-derived BWS6 and BWS9 cells, which have been established from Beckwith-Wiedemann syndrome patients, were kindly provided by Dr M.J. Higgins (Roswell Park Cancer Institute), and Human Wilms' tumor G401 cells were purchased from Human Science (Kyoto, Japan). Both BWS6 and BWS9 cells were maintained in DMEM (Nakarai Tesque, Kyoto, Japan), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Nichirei Bioscience, Tokyo, Japan) and 100 IU/ml of penicillin/streptomycin/glutamine (PSG) (Life Technologies, Carlsbad, CA, USA). G401 cells were cultured in McCoy's 5A (Life Technologies) supplemented with 10% heat-inactivated FBS and 100 IU/ml of PSG. Cells were grown at 37˚C in a CO2 incubator with a humidified atmosphere containing 5% CO2 and 95% air.

Synthesis of PI polyamides targeting human KCNQ1OT1. PI polyamides used in this study were synthesized by a machine-assisted automatic synthesis system, PSSM-8 peptide synthesizer (Shimadzu, Kyoto, Japan) as previously described (23,24). Two PI polyamides termed PI-a and PI-b were designed to span the boundary of 2 of 4 CCAAT boxes within human KCNQ1OT1 promoter region (Fig. 1). Mismatch PI polyamide, which does not recognize CCAAT sequence, was also designed and produced. After synthesis and purification, these PI polyamides were dissolved in ultra pure water at a final concentration of 1 mM as stock solutions.

Gel retardation assay. FITC-labeled oligonucleotides containing target sequences of PI polyamides were synthesized by Eurofin Genomics (Tokyo, Japan). Sequences of the oligonucleotides are as follows: the oligonucleotide containing PI-a target sequence, 5'-GAGTCTAACCAGGTGCCTCTT TGGCCGACCCGGTTAGACTC-3' and the oligonucleotide containing PI-b target sequence, 5'-GGTGTCGACACTA CCGCTTTTGCGGGTTAGTGCAGCACCC-3'. To generate hairpin structure of double-strand DNA, 1 µM of oligonucleotides dissolved in an annealing buffer (5 M NaCl, 500 mM EDTA, 1 M Tris-HCl pH 8.0) were boiled at 100°C for 10 min, and then left at room temperature to gradually cool them down. The resultant FITC-labeled hairpin oligonucleotides were incubated in the presence or absence of 1 or 3 µM of PI polyamides for 1 h at room temperature. The reaction mixtures were separated by electrophoresis through 20% polyacrylamide gel in Tris-borate-EDTA buffer and then visualized with the ImageQuant LAS4000 (Fujifilm, Tokyo, Japan).

Analysis of the intracellular distribution of PI polyamides. BWS6 and G401 cells were maintained in the presence or absence of FITC-labeled PI polyamides for 24 h. After washing in ice-cold phosphate-buffered saline (PBS, Nakarai Tesque), cells were fixed in 4% formaldehyde solution (Wako Pure Chemical Industries, Osaka, Japan) for 30 min, and then stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma-Aldrich, St. Louis, MO, USA). Finally, cells were observed under fluorescence microscope Axiosvert 2000 (Carl Zeiss Microscopy, Oberkochen, Germany).

Quantitative real-time RT-PCR. Seventy-two hours after incubation in the presence or absence of PI polyamides, cells were washed in ice-cold PBS and lysed in TRIzol reagent (Life Technologies). Then, total RNA was prepared according to the manufacturer's instructions, and 500 ng of total RNA was treated with DNaseI (Qiagen, hilden, Germany), followed by reverse transcription using Prime Script (Takara). The resultant cDNA was subjected to quantitative real-time RT-PCR analysis using Dimer Eraser (Takara). The primers used in this analysis are as follows: KCNQ1OT1 (forward: 5'-AATGGG GATGTGAGGAGCAGG3'; reverse: 5'-TGACCCCACTG GAAATATGTCG-3'); GAPDH (forward: 5'-GCACGGTCAA GGTGAAGACGCCAGCAGG-3'; reverse: 5'-TTGGTGAGAAGAGCCACTG GGA-3'). GAPDH was used as an internal control.

Cell proliferation assay. G401 cells were seeded at 2x10⁵ cells/96-well microplates. Then cells were exposed to the indicated concentrations of PI polyamides or left untreated.
At the indicated time points after treatment, cell viability was measured by standard WST-8 assay (Nacalai tesque). Fluorescein-activated cell sorting (FACS). G401 cells were seeded at 5x10^4 cells/2-cm diameter dishes. Then, cells were treated with or without the indicated PI polyamides. Seventy-two hours after treatment, cells were washed in ice-cold PBS, treated with trypsin/EDTA, and 2x10^5 cells were subsequently stained with Annexin V-FITC plus propidium iodide using Annexin V-FITC Apoptosis Detection kit (Abcam, Bristol, UK) according to the manufacturer’s protocols. Finally, cells were sorted by FACSCalibur flow cytometer with FL-1 (Annexin V channels) and FL-2 (PI channels), and then analyzed by CellQuest (BD Biosciences, Rockville, MD, USA). As a positive control, cells were treated with 10 µM of H_2O_2 for 9 h to induce cell death.

Statistical analysis. Statistical significance was calculated using the Student’s t-test. Results were considered as significant with P-value of <0.05.

Results

Specific binding of the PI polyamides to their target double-strand DNA. To verify whether the synthesized PI polyamides (termed PI-a and PI-b) could bind to their target DNA sequences, gel mobility shift assays were performed. For this purpose, the oligonucleotide containing target sequence of PI-a was
incubated with DW, PI-a or non-specific PI polyamide (termed mismatch), and then reaction mixtures were analyzed by polyacrylamide gel electrophoresis. As shown in Fig. 2A, a clearly retarded band was observed in the presence of PI-a but not of mismatch PI polyamide. Similarly, an oligonucleotide/PI-b complex was detectable in the reaction mixture containing PI-b and its target DNA sequence, whereas mismatch PI polyamide failed to bind to oligonucleotide containing the target DNA of PI-b (Fig. 2B). These results indicate that both PI-a and PI-b can specifically recognize and bind to their target DNA sequences in vitro.

**Nuclear distribution of PI polyamides.** Since the PI polyamides could be expected to exert gene silencing function through the direct interaction with their target DNA sequence in cells, it should be required to confirm their nuclear distribution. To adequately address this issue, we generated FITC-conjugated PI-a and PI-b. Human fibroblasts-derived from a BWS patient (BWS6 cells and Wilms' tumor-derived G401 cells were cultured in fresh medium containing PI-a or PI-b. Twenty-four hours after incubation, cells were fixed, stained with DAPI and observed under a confocal microscope. As seen in Fig. 3, a significant nuclear access of FITC-labeled PI-a and PI-b was detected under our experimental conditions. These observations strongly suggest that PI-a and PI-b display nuclear localization without any drug delivery systems such as adenovirus.

**Combinatory treatment with PI-a and PI-b efficiently suppresses KCNQ1OT1 transcription.** Next, we examined whether PI-a and/or PI-b could downregulate the transcription of their target gene KCNQ1OT1. To this end, BWS6, BWS9 and G401 cells were incubated in the presence of the indicated concentrations of PI-a, PI-b, PI-a plus PI-b or mismatch PI polyamide. Seventy-two hours after treatment, total RNA was prepared and the expression level of KCNQ1OT1 was analyzed by quantitative real-time PCR. Unexpectedly, a significant downregulation of KCNQ1OT1 was not detected in all cells examined after PI-a or PI-b single treatment (Fig. 4). Notably, the combinatory treatment of cells with PI-a and PI-b resulted in a marked decrease in the expression level of KCNQ1OT1. As expected, mismatch PI polyamide alone had no detectable effect on the expression level of KCNQ1OT1.

**Reduction in cell viability of G401 cells exposed to a combined treatment with PI-a and PI-b.** Since it has been described that a higher KCNQ1OT1 level is tightly associated with tumor cell growth (25), these observations prompted us to examine whether the simultaneous treatment of PI-a and PI-b could affect cell proliferation of G401 cells. For this purpose, G401 cells were exposed to the indicated concentrations of PI-a plus PI-b or mismatch PI polyamide. At the indicated time points after treatment, cells were subjected to the standard WST8 assay to assess their cell viability. As shown in Fig. 5, the combinatory treatment of PI-a and PI-b caused a remarkable suppression of G401 cell proliferation, whereas mismatch PI polyamide had a negligible effect on their viability. As expected, PI-a or PI-b single treatment did not affect G401 cell proliferation (data not shown). These results imply that the PI polyamide-mediated silencing of KCNQ1OT1 expression might contribute to the suppression of G401 cell proliferation.
G401 cells undergo cell death in response to PI-a plus PI-b. Based on our above-mentioned results, we asked whether a combined treatment with PI-a plus PI-b in G401 cells could induce cell death. To this end, we used PI and Annexin V double staining experiments followed by FACS analysis. It has been established that Annexin V-positive cells undergo early apoptotic cell death (26). G401 cells were treated with the indicated concentrations of PI polynamide (PI-a, PI-b or PI-a plus PI-b) or left untreated. Seventy-two hours after treatment, total RNA was prepared from these cells and the expression level of KCNQ1OT1 was examined by quantitative real-time RT-PCR. *P<0.01.

Discussion

In the present study, we successfully produced pyrrole-imidazole (PI) polynamides (PI-a and PI-b) against CCAAT boxes within the promoter region of oncogenic KCNQ1OT1 gene. Based on our present results, the combined treatment with PI-a and PI-b in Wilms’ tumor-derived G401 cells significantly reduced KCNQ1OT1 gene expression and also their cell viability, which might be caused by the induction of apoptosis. The molecular targeting anticancer drugs, which could block oncogenic signaling pathways, would be powerful and promising cancer therapeutics. Recent high-throughput technologies make it much easier to identify critical genes implicated in the initiation and/or progression of a variety of cancers, which are aberrantly expressed and/or activated in cancer cells (28). Although several antisense oligo DNA and siRNA against these cancer-related specific genes have been developed, their poor cellular uptake and also delivery to the appropriate organs or tissues remain major obstacles for their clinical application (29). Since it has been shown that PI polynamides are efficiently localized into cell nucleus without any specific drug delivery systems such as virus vehicles both in vitro and in vivo (30,31), these observations prompted us to develop gene-specific silencing PI polynamides. For example, we have previously described that PI polynamide recognizing AP-1-binding site located within the promoter region of matrix metalloproteinase (MMP)-9 gene massively reduces cells were barely detectable in untreated or mismatch-treated cells. In contrast, substantial amounts of cells were enriched in early and late apoptotic fractions. Average number of cells showing early apoptosis, late apoptosis or necrosis is depicted as a graph in Fig. 6B. According to our present results, the combinatory treatment of cells with PI-a and PI-b markedly induced apoptosis in a dose-dependent manner. In addition, the relatively small number of Annexin V-negative/PI-positive cells was observed in each experiment.
its expression level and also inhibits malignant phenotypes of cancer cells both in vitro and in vivo (30).

In our results, single application of either PI-a or PI-b did not affect the expression level of KCNQ1OT1, however, the treatment with the combination of these two PI polyamides downregulated KCNQ1OT1. At the present moment, we could not find good explanation for this discrepancy. Previously, Du et al reported that an introduced point mutation in each of four CCAAT box sequences reduced promoter activity modestly but not completely (10,32). The authors also showed data suggesting that simultaneous targeting of two CCAAT box regions of KCNQ1OT1 promoter could be also involved in the transcriptional regulation of this gene. Those findings indicate that blocking just one CCAAT box is not enough to suppress the gene expression level.

Of note, we found for the first time that the combinatory treatment of our PI polyamides significantly downregulates oncogenic KCNQ1OT1 gene transcription, and its downregulation leads to the suppression of cancerous cell proliferation as well as the induction of apoptosis. It has been shown that KCNQ1OT1 has the ability to regulate the imprinting status of the circumjacent genes, such as ASCL2, TSPAN32, CD81, TSSC4, p57KIP2, SLC22A18 and PHLDA2 of the same allele (13,33). Among them, p57KIP2 has been considered to be one of tumor suppressor gene products implicated in BWS (34). Accumulating evidence suggests that p57KIP2 is one of Cip/Kip family of cyclin-dependent protein kinase inhibitor and blocks cell proliferation by inhibiting cell cycle progression (35). Additionally, p57KIP2 has the ability to promote apoptosis and prohibit tissue invasion as well as metastasis (36,37). Therefore, we sought to examine whether the combinatory treatment of the PI polyamides could affect the expression level of the above-mentioned circumjacent genes including p57KIP2. Unexpectedly, the expression level of these genes remained unchanged even in the presence of the PI polyamides (data not shown). At present, we do not know how the combined treatment with the two PI polyamides could suppress cancerous cell proliferation and also induce apoptosis. Further study is required to adequately address this issue.

Collectively, our present results indicate that the above reported PI polyamides targeting KCNQ1OT1 promotes apoptosis in Wilms' tumor G401 cells through a significant downregulation of KCNQ1OT1 transcription, and support our hypothesis that PI polyamide-mediated gene silencing might be a novel attractive strategy for anticancer therapy.

Acknowledgements

This work was supported by the Academic Frontier Project for 2006 Project for Private Universities, a matching fund subsidy from MEXT (to H.N.), and MEXT-Supported Program for

References


