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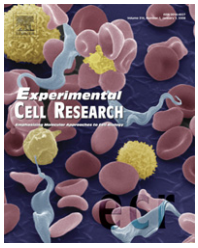

Research Article

Epigenetic and HIF-1 regulation of stanniocalcin-2 expression in human cancer cells

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ABSTRACT

Mammalian stanniocalcin-2 (STC2) is a secreted glycoprotein hormone with a putative role in unfolded protein response and apoptosis. Here we reported that STC2 expression was sporadically abrogated in human cancer cells by transcriptional silencing associated with CpG island promoter hypermethylation. Direct sequencing of bisulfite-modified DNA from a panel of seven human cancer cell lines revealed that CpG dinucleotides in STC2 promoter was methylated in human ovarian epithelial cancer (SKOV3, OVCAR3 and CaOV3), pancreatic cancer (BxP3), colon adenoma (HT29), and leukemia (Jurkat cells). STC2 CpG island hypermethylation was accompanied with a low basal STC2 expression level. Treatment of these cancer cells with 5-aza-2′-deoxycytidine (5-aza-CdR), an inhibitor of DNA methylation significantly induced STC2 expression. Using SKOV3 cells as a model, the link between DNA demethylation and STC2 expression was consistently demonstrated with hydralazine treatment, which was shown to reduce the protein level of DNA methyltransferase 1 (DNMT1) but stimulated STC2 expression. Two human normal surface ovarian cell-lines (i.e. IOSE 29 and 398) showed no methylation at CpG dinucleotides in the examined promoter region and were accompanied with high basal STC2 levels. Hypoxia stimulated STC2 expression in SKOV3 cells was markedly increased in 5-aza-CdR pretreated cells, showing that DNA methylation may hinder the HIF-1 mediated activation. To elucidate this possibility, RNA interference studies confirmed that endogenous HIF-1α was a key factor for STC2 gene activation as well as in the synergistic induction of STC2 expression in 5-aza-CdR pretreated cells. Chromatin immunoprecipitation (ChIP) assay demonstrated the binding of HIF-1α to STC2 promoter. The binding was increased in 5-aza-CdR pretreated cells. Collectively, this is the first report to show that STC2 was aberrantly hypermethylated in human cancer cells. The findings demonstrated that STC2 epigenetic inactivation may interfere with HIF-1 mediated activation of STC2 expression.

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Introduction

Mammalian stanniocalcins (STC1), an orthology of fish STC1, is a glycosylated protein hormone that was cloned in the screening process of investigating cancer-related genes [1]. Human STC1 has recently been identified as a proapoptotic factor, and is found to be activated by HIF-1 in human cancer cells and tissues [2–4]. Its paralog, STC2 was identified by searching EST databases for DNA sequences related to STC1 [5–8]. The relatedness of STC2 to STC1 is greatest at the N-terminus. STC2 protein is widely expressed in mammalian tissues, however its receptor is not yet identified. There is growing evidence to show that altered expression of STC2 has potential roles in cancinogenesis, including breast and ovarian cancers [9–11]. Its expression is found to be associated with estrogen receptors in human breast cancer [9]. The prognostic significance of STC2 expression in breast cancer was suggested [12,13]. STC2 is recently found to be a novel target in unfolded protein response in mammalian cells and the siRNA or overexpression of STC2 showed a modest cytoprotective effect on thapsigargin-induced cell death [14]. Transgenic mice overexpressing human STC2 revealed the protein may act as a growth inhibitor, leading to growth retardation in the animals [15]. In human, STC2 is encoded by a single copy gene localized at human chromosome 5q35.1 [16,17]. It has been reported that chromosome 5q contains regions that influence tumor progression when deleted. Hitherto limited information is available on the regulation and function of STC2.

In normal mammalian cells, CpG islands at the promoters of actively transcribed genes are mostly unmethylated. Deregulation of genomic DNA methylation is found to be associated with carcinogenesis [18]. Transcriptional silencing by hypermethylation of CpG dinucleotides in promoter region is an alternative and emergent mechanism for the inactivation of tumor-suppressor genes [18,19]. In this report, we analyzed the methylation status of 5′-CpG islands of STC2 gene in several human cancer cell-lines. The influences of DNA methylation on STC2 expression in cancerous and noncancerous human ovarian cell lines were examined. We report that STC2 undergoes CpG island methylation-associated gene silencing in human cancer cells. The epigenetic loss of STC2 function can be rescued by the use of an DNA demethylating agent (5-aza-CdR) or a methyltransferase inhibitor (hydralazine). We found that the STC2 promoter was aberrantly hypermethylated that may reduce its responsiveness to transcriptional activation by HIF-1. Hypoxia treatment of 5-aza-CdR pre-treated cells superinduced STC2 expression as compared to hypoxia treatment alone. The superinduction was HIF-1α dependent as revealed by HIF-1α silencing and ChIP assays. In this study, we propose that promoter hypermethylation is a potential mechanism controlling STC2 expression in human tumor cells.

Materials and methods

Human cancer cells lines

Seven human cancer cell lines were obtained from ATCC and two immortalized human normal ovarian surface epithelial cell-lines (OSE) were provided by Dr. AS Wong (Department of Zoology, The University of Hong Kong). These included tumors of ovary (SKOV3, OVCAR-3, CaOV3), colon (HT-29, CaCo-2), lymphoblastic leukemia (Jurkat), pancreatic (BxP3) and the normal ovarian surface epithelial cells (i.e. IOSE 29 and 398). Cells were grown and maintained in their respective growth media supplemented with 10% FBS. Genomic DNA was extracted with the DNeasy Tissue kit (Qiagen), for bisulfite DNA sequencing. Total RNA was extracted with TRIzol and was subjected to cDNA syntheses and real-time PCR assay.

Analysis for CpG islands in the human STC2 promoter and bisulfite DNA sequencing

Genomic DNA was modified with sodium bisulfite using Cp Genome DNA Modification kit (Chemicon International) and was used in PCR for bisulfite sequencing. A 2374 nt fragment of the human STC2 promoter, containing the translation start site, was selected from the National Center for Biotechnology Information database using the accession number NT 023133. This sequence was then analyzed with MethPrimer, using the following settings: Island size >200 nt, GC percent >50, and observed/expected CpG ratio >0.6. Two CpG islands with sizes of 1095 nt (CpG-2) and 643 nt (CpG-1) were identified. DNA methylation was analyzed by bisulfite genomic sequencing of the 381 nt at CpG-2 of the human STC2 promoter. Both strands of the template were sequenced using the following primers GGAGGAGATGGAGATAATTTTTT-forward and AAAACC-CAAAACAACCCTAAATC-reverse. Methylation-specific PCR (MSP) was conducted using specific primers for the methylated or unmethylated bisulfite-modified DNA. The primers were unmethylated sense 5′-TGGGAATTTTATTGAATTTTAAATG-3′ and unmethylated antisense 5′-CAAATACTCCAAAA-CAATAATCAAA-3′; methylated sense 5′-TTGGGAATTTTATT-GAATTTTA AAC-3′ and methylated antisense 5′-TACGAATACTCCAAAACAATAATCG-3′; unmethylated sense 5′-GTATAAAAGATTTAAGGTTGTGTGG-3′ and unmethylated antisense 5′-CTTCAAAATAAACTTCCCCTACACT-3′; methylated sense 5′-TTGTATA AAAGATTTAAGGTTGCGC-3′ and methylated antisense 5′-TCTTCAAAATAAACTT CCCCTACG-3′. Primers for amplification were designed with MethPrimer. PCRs were conducted at 94 °C for 5 min, 35 cycles of denaturing (94 °C for 30 s), annealing (56 °C for 1 min), and extension (72 °C for 1 min) followed by 10 min final extension. For bisulfite DNA sequencing, PCR product was resolved by electrophoresis on a 2% agarose gel and was gel purified for direct DNA sequencing by an outside vendor (TechDragon, Hong Kong). For MSP, PCR products were resolved in 1% agarose gels that were stained with ethidium bromide and visualized under UV light.

Effects of 5-aza-2′-deoxycytidine (5-aza-CdR) and hydralazine on STC2 mRNA levels

Two human normal surface ovarian cell-lines (i.e. IOSE 29 and 398) and the seven human cancer cells grown in their respective growth media were split individually on day 0 and treated with 2 μM 5-aza-2′-deoxycytidine (5-aza-CdR, Sigma) on day 1. The cells were incubated in 5% CO_2 at 37 C. Culture medium was changed daily and freshly prepared 5-aza-CdR was added. Total RNA were isolated on day 4 for real-time PCR assay to determine the expression level of STC2 mRNA.

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