Kaiso Contributes to DNA Methylation-Dependent Silencing of Tumor Suppressor Genes in Colon Cancer Cell Lines

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Abstract

Aberrant CpG methylation of tumor suppressor gene regulatory elements is associated with transcriptional silencing and contributes to malignant transformation of different tissues. It is presumed that methylated DNA sequences recruit repressor machinery to actively shutdown gene expression. The Kaiso protein is a transcriptional repressor expressed in human and murine colorectal tumors that can bind to methylated clusters of CpG dinucleotides. We show here that Kaiso represses murine colorectal tumors that can bind to methylated clusters of CpG dinucleotides. The contribution of Kaiso to epigenetic silencing was underlined by the fact that Kaiso depletion induced tumor suppressor gene expression without affecting DNA methylation levels. As a consequence, colon cancer cells became susceptible to cell cycle arrest and cell death mediated by chemotherapy. The data suggest that Kaiso is a methylation-dependent “opportunistic” oncogene that silences tumor suppressor genes when they become hypermethylated. Because Kaiso inactivation sensitized colon cancer cell lines to chemotherapy, it is possible that therapeutic targeting of Kaiso could improve the efficacy of current treatment regimens. [Cancer Res 2008;68(18):7258–63]

Introduction

Malignant transformation of normal tissues is a consequence of both genetic and epigenetic lesions. The former result from mutations or gain or loss of DNA sequence, whereas the latter result from changes in cytosine (CpG) methylation or DNA-associated proteins. Methylation of CpG dinucleotides clustered in regulatory elements is well established as a mechanism of epigenetic silencing (1). Similar to genetic lesions, epigenetic lesions in the form of specific DNA methylation patterns can be inherited and cause cancer predisposition in families. For example, inherited loss of allele-specific epigenetic silencing of the IGF2 locus can predispose to renal and intestinal cancer (2). More commonly, aberrant epigenetic silencing is a consequence of somatic redistribution of DNA methylation, which increases in likelihood during aging and is accelerated by environmental factors, such as diet or the presence of chronic inflammatory states (e.g., ref. 3).

Epigenetic silencing is a contributing factor to the pathogenesis of colorectal cancer. Loss of DNA methyltransferase activity attenuates intestinal tumorigenesis in animals with mutant APC or deficient in mismatch repair (e.g., refs. 4, 5). Genes silenced by hypermethylation in colon cancer include important tumor suppressor or DNA repair genes, such as CDKN2A (p16/INK4A), HIC1, MGMT, and others (6). Moreover, a specific CpG methylator phenotype has been identified in colon cancer, which is associated with BRAF mutations and seems to account for most cases of acquired microsatellite instability (7). Reversal of epigenetic silencing is therefore a potentially desirable modality of targeted therapy for cancer, and DNA methyltransferase inhibitors have been shown to be effective in certain types of tumors (8).

The principal mechanism through which methylated DNA is silenced is via recruitment of methylCpG-binding transcription factors (9). Two classes of such proteins have been identified—the methylCpG binding domain (MBD) family and the Kaiso subfamily of C2H2 zinc finger proteins (10). Kaiso is a ubiquitous protein that binds to clusters of methylated CpG dinucleotides with 10-fold higher affinity than the MBDS and represses transcription at least in part by recruiting the N-CoR corepressor (11, 12). Loss of Kaiso resulted in gastrulation defects in lower vertebrates but had no obvious developmental phenotype in mice (13–15). Kaiso protein levels were increased in murine intestinal cancer versus matched normal mucosa and were also expressed in human colon cancer tissue samples (15). Loss of Kaiso resulted in an attenuation of the intestinal neoplasia phenotype on the APC mutant (APCmin) mouse background (15). Here, we show that Kaiso binds to methylated tumor suppressor and DNA repair genes in colon cancer cells. Loss of Kaiso releases these genes from silencing, although they remained methylated, suggesting that methylated genes cannot be silenced in these cells in the absence of Kaiso. Reactivation of these genes restored cellular checkpoints and made cells susceptible to stress challenges. These results suggest that Kaiso is a methylation-dependent oncogene that provides a survival advantage to colon cancer cells.

Materials and Methods

Western blot. Colo201, Colo205, Colo320, DLD1, HCT115, HCT116, and SW48 were kind gifts of Dr. John Mariadason, Montefiore Medical Center. Kaiso was detected in cell lysates by immunoblotting, as described in
Kaiso represses methylated tumor suppressor genes in colon cancer cells. Our previous results suggested that Kaiso plays an important role in colon cancer cells. We used the Kaiso small interfering RNA (siRNA) knockdown strategy to investigate the role of Kaiso in colon cancer cells. siRNA transfections were performed using Lipofectamine 2000 (Invitrogen) and detected by SybrGreen (Applied Biosystems) on an Opticon2 thermal cycler (DNA-Technology, Inc.). Each reaction was amplified by end point PCR or SYBR green qRT-PCR on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). MassArray mass spectrometry was used to detect the presence of methylated tumor suppressor genes.

**Results and Discussion**

Kaiso represses methylated genes in colon cancer cells. Our previous results suggested that Kaiso plays an important role in regulating the expression of methylated tumor suppressor genes in colon cancer cells. We used the Kaiso siRNA knockdown strategy to investigate the role of Kaiso in colon cancer cells. siRNA transfections were performed using Lipofectamine 2000 (Invitrogen) and detected by SybrGreen (Applied Biosystems) on an Opticon2 thermal cycler (DNA-Technology, Inc.). Each reaction was amplified by end point PCR or SYBR green qRT-PCR on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). MassArray mass spectrometry was used to detect the presence of methylated tumor suppressor genes.

**Figure 1.** Kaiso represses methylated genes in colon cancer cells. The mRNA abundance of the Kaiso, CDKN2A, MGMT, and HIC1 was detected by qRT-PCR in Colo320 (A) or HCT116 (B) cells 48 h after transfection with Kaiso siRNA1 (black columns) or a Kaiso siRNA 2 (gray columns). mRNA abundance was represented as the fold change $\Delta^{\text{Ct}}$ of each mRNA relative to HPRT and compared with control siRNA transfected cells (which is set at a value of 1).

Each sample was cleaned with a Qiagen PCR purification kit (50 μL of elution buffer). All precipitated DNA was divided into three parts. Each part was subject to enzymatic digestion with MspI, HpaII, or bovine serum albumin, respectively. Each sample was incubated with endonuclease (10 units) overnight followed by inactivation at 65°C for 20 min. The products were amplified by end point PCR or SYBR green qRT-PCR on a DT-96x v6.1 thermal cycler (DNA-Technology, Inc.). Each reaction was performed in triplicate. Restriction mix at 1/10 V was used for each reaction. All ChIP primers are shown in Supplementary Table S3.

**MassArray.** Quantification of DNA methylation was carried out by MALDI-TOF mass spectrometry using Epityper by MassArray on bisulfite-converted DNA, as previously described (17). MassArray primers were designed to cover two regions upstream of the transcription start site of the CDKN2A gene, spanning chr9:21,966,178-21,966,699 and chr9:21,964,659-21,964,922 (primer sequences shown in Supplementary Table S3).

**Cell viability and death.** Colo320, HCT116, and CDD-18Co cells untransfected or transfected with Kaiso siRNA or negative control siRNA were starved for 24 h; after that, they were treated with 50 μM of etoposide. Colonic fibroblasts are more sensitive to etoposide and were also cultured in 10 μM of concentration. The cells were harvested at the indicated time points, and cell viability and death were assessed using fluorescence microscopy by mixing 2 μL of acridine orange (100 μg/mL), 2 μL of ethidium bromide (100 μg/mL), and 20 μL of the cell suspension. A minimum of 200 cells was counted in at least five random fields. Live apoptotic cells were differentiated from dead apoptotic, necrotic, and normal cells by examining the changes in cellular morphology on the basis of distinctive nuclear and cytoplasmic fluorescence. Viable cells display intact plasma membrane (green color), whereas dead cells display damaged plasma membrane (orange color). An appearance of ultrastructural changes, including shrinkage, heterochromatin condensation, and nuclear degranulation, are more consistent with apoptosis and disrupted cytoplasmic membrane with necrosis.

**Propidium iodide staining.** Cells treated as above were fixed in cold 70% ethanol for at least 30 min at 4°C and washed twice. Fifty microliters of propidium iodide at 1 mg/mL (Sigma-Aldrich) and 20 μL of RNase-A at 10 μg/mL (Sigma-Aldrich) were then added, after which the cells were analyzed by flow cytometry in a FACScan (BD Biosciences). Cell cycle profiles were determined using Cell Quest software (BD Biosciences).

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1. ref. 15, using the Kaiso 6F monoclonal antibody (Upstate Biotechnology) and our Kaiso zinc finger polyclonal antibody (15). For loading control, immunoblotting were also performed with actin antibodies (C11, Santa Cruz Biotechnology). Quantitation of bands was performed using ImageJ software. The results are shown normalized to actin and relative to control.

2. Kaiso small interfering RNA (siRNA) knockdown. Colo320 and HCT116 human colorectal carcinoma cell lines and human colonic fibroblasts CDD-18Co cells (American Type Culture Collection CRL-1459) were plated onto 10-cm dishes and incubated overnight (37°C, 5% CO2) to have an approximate confluence of 60% to 70% at the time of transfection. All small interfering RNA (siRNA) transfections were performed using Lipofectamine 2000 (Invitrogen). The assay was performed using 100 nmol/L of specific Kaiso oligonucleotide siRNA or an irrelevant oligonucleotide sequence (from Ambion). Cells were collected after 24, 48, or 72 h, depending on the experiment to perform, mRNA extraction, protein analysis, apoptosis determination, and cell cycle study. For siRNA sequences, see Supplementary Table S1.

3. Real time-PCR. RNA was extracted from Colo320, HCT116, and CDD-18Co cells using TRIzol (Invitrogen Corp.). cDNA was prepared using Superscript III First Strand cDNA Synthesis kit (Invitrogen) and detected by SybrGreen (Applied Biosystems) on an Opticon2 thermal cycler (MJ Research). We normalized gene expression to hypoxanthine phosphoribosyltransferase (HPRT) and expressed values subject to enzymatic digestion with MspI, HpaII, or bovine serum albumin, respectively. Each sample was incubated with endonuclease (10 units) overnight followed by inactivation at 65°C for 20 min. The products were amplified by end point PCR or SYBR green qRT-PCR on a DT-96x v6.1 thermal cycler (DNA-Technology, Inc.). Each reaction was performed in triplicate. Restriction mix at 1/10 V was used for each reaction. All ChIP primers are shown in Supplementary Table S3.

4. Chromatin immunoprecipitation assay. Quantitative chromatin immunoprecipitation assay (ChIP) was performed, as previously reported (16), but in this case, with Colo320 and HCT116 cells using either ZFh6 rabbit polyclonal Kaiso antibody or an IgG isotype control. DNA samples were cleaned using a Qiagen PCR purification kit (50 μL of elution buffer). All precipitated DNA was divided into three parts. Each part was subject to enzymatic digestion with MspI, HpaII, or bovine serum albumin, respectively. Each sample was incubated with endonuclease (10 units) overnight followed by inactivation at 65°C for 20 min. The products were amplified by end point PCR or SYBR green qRT-PCR on a DT-96x v6.1 thermal cycler (DNA-Technology, Inc.). Each reaction was performed in triplicate. Restriction mix at 1/10 V was used for each reaction. All ChIP primers are shown in Supplementary Table S3.

5. Cell viability and death. Colo320, HCT116, and CDD-18Co cells untransfected or transfected with Kaiso siRNA or negative control siRNA were starved for 24 h; after that, they were treated with 50 μM of etoposide. Colonic fibroblasts are more sensitive to etoposide and were also cultured in 10 μM of concentration. The cells were harvested at the indicated time points, and cell viability and death were assessed using fluorescence microscopy by mixing 2 μL of acridine orange (100 μg/mL), 2 μL of ethidium bromide (100 μg/mL), and 20 μL of the cell suspension. A minimum of 200 cells was counted in at least five random fields. Live apoptotic cells were differentiated from dead apoptotic, necrotic, and normal cells by examining the changes in cellular morphology on the basis of distinctive nuclear and cytoplasmic fluorescence. Viable cells display intact plasma membrane (green color), whereas dead cells display damaged plasma membrane (orange color). An appearance of ultrastructural changes, including shrinkage, heterochromatin condensation, and nuclear degranulation, are more consistent with apoptosis and disrupted cytoplasmic membrane with necrosis.

6. Propidium iodide staining. Cells treated as above were fixed in cold 70% ethanol for at least 30 min at 4°C and washed twice. Fifty microliters of propidium iodide at 1 mg/mL (Sigma-Aldrich) and 20 μL of RNase-A at 10 μg/mL (Sigma-Aldrich) were then added, after which the cells were analyzed by flow cytometry in a FACScan (BD Biosciences). Cell cycle profiles were determined using Cell Quest software (BD Biosciences).
Kaiso binds to methylated promoters in colon cancer cells. Quantitative ChIP assays were performed in Colo320 (A) or HCT116 cells (B) cells to detect Kaiso binding to the CDKN2A, MGMT, and HIC1 promoters, as indicated. The fold enrichment versus input of Kaiso antibodies (gray columns) versus IgG control (black columns) is shown on the Y axis. C, end point Kaiso ChIP was performed in HCT116 cells, in which CDKN2A is hemimethylated (lane 1). Lane 2, PCR of ChIP products after digestion with MspI; lane 3, PCR of ChIP products after digestion with HpaII (methylation resistant) shows that Kaiso binds to the methylated allele; lanes 4 and 5, IgG control and input, respectively. D, ChIP similar to C was performed, but the products were amplified by real-time PCR. The Y axis indicates the fold difference in amplicons abundance in each channel compared with MspI digested sample. The input is shown in gray. Digestion with HpaII caused a partial decrease in amplicons abundance compared with undigested DNA due to loss of the unmethylated allele. The Kaiso IP is shown in black columns. In this case, the HpaII digest is equivalent to the undigested control, because Kaiso presumably does not bring down the unmethylated allele.

In vivo in facilitating tumorigenesis of intestinal neoplasia in mice (15). Because this phenotype was similar to that reported in animals deficient in the MBD2 methylation-dependent repressor (18) and Kaiso binds with high affinity to methylated DNA (11), we wondered whether Kaiso might mediate the observed effect by repressing methylated genes in colon cancer cells. We previously showed that Kaiso is also expressed in human colon cancer patient samples (15), and we now report that Kaiso is expressed in a number of human colon cancer cell lines, including Colo201, Colo205, Colo320, DLD1, HCT115, HCT116, and SW48 (Supplementary Fig. S1). Colo320 and HCT116 cells were further examined by methylation assay to measure the abundance of DNA methylation present at the promoters of 93 genes, including a number of loci previously associated with aberrant hypermethylation in various tumors (Supplementary Fig. S2). Hypermethylation was observed in a number of genes in Colo320 (57% of genes) and HCT116 cells (24% of genes). The CDKN2A, HIC1, and MGMT genes were hypermethylated in both cell lines and selected for further analysis as possible Kaiso targets.

CDKN2A normally plays a critical tumor suppressor role by inactivating cyclin D–cdk4/6 complexes, blocking Rb hyperphosphorylation and, thus, causing cell cycle arrest (19). HIC1 encodes for a BTB/POZ domain transcriptional repressor in the same family as Kaiso, and can modulate p53 damage responses through regulation of SIRT1 (20). HIC1 deficiency was associated with the development of multiple tumors in mice (21). Epigenetic silencing of MGMT can cause defects in DNA mismatch repair and contribute to genomic instability (reviewed in ref. 6). We wished to know whether these hypermethylated genes were repressed by Kaiso. Therefore, we performed Kaiso depletion studies using two different siRNA sequences in both Colo320 and HCT116 cells followed by qRT-PCR to measure the abundance of CDKN2A, HIC1, and MGMT mRNA. Nucleofection of either Kaiso siRNA molecule readily down-regulated Kaiso mRNA (Fig. 1A and B) and protein (Supplementary Fig. S3). Kaiso depletion induced expression of all three of these transcripts compared with HPRT, with the exception that one of the siRNA duplexes failed to induce HIC1 in HCT116 (Fig. 1A and B).

**Kaiso binds directly to hypermethylated genes.** To determine whether Kaiso was directly binding to these methylated genes, ChIP primers were designed to amplify the Kaiso (mCmCG) binding sites in the promoter CpG islands of these genes. Immunoprecipitation with polyclonal Kaiso antibodies (but not control IgG) enriched these fragments of the CDKN2A, HIC1, and MGMT promoters in both HCT116 and Colo320 cells in quantitative ChIP assays (Fig. 1A and B). In HCT116 cells, CDKN2A is inactivated by hypermethylation of one allele and mutation of the other (which is not methylated; ref. 22). To determine whether Kaiso was binding to the methylated allele, the products of Kaiso ChIP from HCT116 cells were subjected to digestion with the isoschizomer endonucleases MspI and HpaII. Both enzymes digest the sequence CCGG, but HpaII is blocked by CpG methylation. Accordingly, MspI digestion destroyed the entire pool of enriched CDKN2A sequence, but Kaiso enriched fragments were at least partially resistant to HpaII (Fig. 2C). When the experiment was repeated using real-time PCR amplification, the products could be measured more precisely (Fig. 2D). Under these conditions, HpaII digestion only partially depletes input DNA, which consists of both the methylated and unmethylated alleles. In contrast, HpaII digest of Kaiso enriched CDKN2A fragments is undistinguishable from undigested DNA, because Kaiso pull down enriches only for the...
methylated allele. These results show that Kaiso can bind and represses methylated tumor suppressor genes in colon cancer cells.

**Kaiso depletion does not reverse aberrant hypermethylation.** The fact that Kaiso depletion can reactivates loci silenced by methylation suggests that methylation alone is insufficient to repress expression of these genes. Reciprocally, these results suggest that DNA methylation is insufficient to silence these methylated genes in the absence of Kaiso. To determine whether this is the case, we performed MassArray epityping of two regions of the CDKN2A promoter (Fig. 3). These corresponded to a portion of the promoter CpG island (Chr9 21,964,659–21,964,922) containing two Kaiso methylCpG binding sites and to a sequence upstream of the CpG island (Chr9 21,966,178–21,966,699), which is not associated with hypermethylation as a negative control (Fig. 3). MassArray analysis provides a quantitative measurement of the percentage of methylation of most or all CpGs contained within a given sequence. In this case, all of the 26 CpG dinucleotides tested, including those corresponding to the Kaiso binding site, in the CGI region were 100% methylated in Colo320 cells and 50% methylated in HCT116 cells (Fig. 3). This is consistent with the known pattern of methylation in HCT116 cells, as mentioned above (22). In contrast, most of the upstream (non-CGI) sequence was unmethylated. Kaiso depletion did not affect the percentage methylation of any of these CpGs in either fragment, demonstrating that promoter CpG methylation at the CDKN2A locus is insufficient to induce transcriptional silencing in the absence of Kaiso binding. These findings were also confirmed by bisulfite pyrosequencing (Supplementary Figs. S4 and S5). The results suggest that tumor-related promoter methylation is insufficient on its own to silence expression of the affected genes, the repression of which are thus at least partially dependent on the continued presence of Kaiso. These data are consistent with the emerging notion that aberrant DNA methylation may not have intrinsic silencing properties. In accordance with this view, siRNA-induced depletion of MBD2 protein in cancer cells could also reactivate methylated genes without demethylation (23, 24). Histone deacetylases also contribute to silencing methylated DNA, as exemplified by the fact that class III histone deacetylase SIRT1 could also rescue methylated genes from silencing without affecting DNA methylation levels (25) and treatment of breast cancer cell lines with a histone deacetylase inhibitor could reactivate methylated estrogen receptor gene without inducing demethylation (26). Taken together, these data suggest that silencing of aberrantly methylated genes is mediated through several mechanisms, and further research is warranted to determine whether these different factors act cooperatively or distribute preferentially to different sites in the genome.

**Kaiso depletion sensitizes colon cancer cells to cell cycle arrest and cell death.** Failure to induce tumor suppressor-mediated cellular checkpoints contributes to tumor cell resistance to chemotherapy (27). Therefore, we wondered whether Kaiso depletion would cause colon cancer cells to become more susceptible to environmental stress or cytotoxic drugs. At 24 hours after transfection of two Kaiso siRNA or negative control sequence, HCT116 and Colo320 cells were serum starved and evaluated for effects in cell cycle progression by propidium iodide flow cytometry at 24 and 48 hours (Fig. 4 and B). Untransfected (not shown) and control transfected Colo320 cells exhibited 51% and 57% of cells in the G1 fraction at 24 and 48 hours, respectively. Kaiso depleted Colo320 cells showed 68% or 62% at 24 hours and 71% or 76% at 48 hours for the two different siRNAs, respectively. Fifty-one percent
of untransfected (not shown) or control transfected HCT116 cells were in G1 phase at 24 and 48 hours, whereas 61% and 66% of Kaiso depleted cells were arrested in G1 at 24 hours and 78% and 56% were decreased at 48 hours (Fig. 4A and B). Both cell lines were also exposed to 50 μmol/L etoposide after Kaiso depletion transfection examined for evidence of cell death by acridine orange/ethidium bromide staining. Control or untransfected Colo320 cells exhibited up to 10% or 11% cell death at 48 and 72 hours, respectively; 20-23%
of Colo320 cells were dead at 48 hours, and 25% to 35% were dead at 72 hours. Control or untransfected HCT116 cells exhibited no >5% cell death after 48 or 72 hours (Fig. 4C). In contrast, 28% and 22% of Kaiso-depleted HCT116 cells were dead at 48 hours, and 27% and 24% were dead at 72 hours with the two different siRNAs, respectively (Fig. 4D). In contrast, Kaiso depletion in normal intestinal fibroblasts (CCD-18Co) cells did not induce expression of CDKN2A, MGMT, or HIC1 and did not sensitize these cells to killing by etoposide nor alter their cell cycle profiles (Supplementary Fig. S6). Taken together these results suggest that by repressing methylated promoters for genes involved in cell cycle and survival checkpoints, Kaiso facilitates tumor cell proliferation and resistance to chemotherapeutic agents.

The fact that Kaiso depletion was detrimental to tumor growth and survival both in vitro and in an animal model strongly suggests that Kaiso facilitates tumorigenesis in mammalian cells. This is a key point from a therapeutic standpoint. DNA methyltransferase inhibitor (MTI) drugs are currently used and investigated for cancer therapy. MTIs can cause DNA damage both by incorporating into DNA and through extensive chromosomal hypomethylation, which may lead to genomic instability and tumorigenesis (28). In contrast, loss of Kaiso did not cause developmental defects or cancer predisposition (15). Instead, loss of Kaiso renders tumor cells more susceptible to chemotherapy drugs, possibly by reactivating physiologic cellular checkpoints. These biological effects are likely combinatorial and due to loss of silencing of a large cohort of methylated genes, although it seems possible that reactivation of CDKN2A and HIC1 are involved. Kaiso was also recently shown to directly repress the retinoblastoma gene when it becomes methylated (29). Taken together, therapeutic targeting of Kaiso might thus enhance the sensitivity of tumor cells to cytotoxic drugs without causing genome-wide disruptions. One way this could be achieved is by targeting the Kaiso BTB domain, which can recruit the N-CoR corepressor to mediate transcriptional repression (12). We recently showed that peptidomimetics blocking the association of the SMRT and N-CoR corepressors to the BTB/POZ oncogene BCL6 could specifically overcome its tumorigenic properties (30). A similar strategy could be successful in targeting Kaiso.

Disclosure of Potential Conflicts of Interest

P.W. Laird: Epigenomics, AG. The other authors disclosed no potential conflicts of interest.

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